the FEBS Journal

Proline-serine-threonine phosphatase interacting protein 1 inhibition of T-cell receptor signaling depends on its SH3 domain

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Keywords
proline-serine-threonine phosphatase interacting protein 1 (PSTPIP1); PTPN22; pyogenic arthritis, pyoderma gangrenosum and acne (PAPA); T cell; T-cell receptor

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(Received 19 February 2014, revised 19 May 2014, accepted 4 July 2014)

doi:10.1111/febs.12912

Proline-serine-threonine phosphatase interacting protein 1 (PSTPIP1) is an adaptor protein associated with the cytoskeleton that is mainly expressed in hematopoietic cells. Mutations in PSTPIP1 cause the rare autoinflammatory disease called pyogenic arthritis, pyoderma gangrenosum, and acne. We carried out this study to further our knowledge on PSTPIP1 function in T cells, particularly in relation to the phosphatase lymphoid phosphatase (LYP), which is involved in several autoimmune diseases. LYP–PSTPIP1 binding occurs through the C-terminal homology domain of LYP and the F-BAR domain of PSTPIP1. PSTPIP1 inhibits T-cell activation upon T-cell receptor (TCR) and CD28 engagement, regardless of CD2 costimulation. This function of PSTPIP1 depends on the presence of an intact SH3 domain rather than on the F-BAR domain, indicating that ligands of the F-BAR domain, such as the PEST phosphatases LYP and PTP-PEST, are not critical for its negative regulatory role in TCR signaling. Additionally, PSTPIP1 mutations that cause the pyogenic arthritis, pyoderma gangrenosum and acne syndrome do not affect PSTPIP1 function in T-cell activation through the TCR.

Structured digital abstract

- LYP physically interacts with PSTPIP1 by pull down (View interaction)
- PSTPIP1 physically interacts with LYP by anti tag coimmunoprecipitation (1, 2, 3, 4)
- LYP physically interacts with PSTPIP1 by anti bait coip (View interaction)

Introduction

Proline-serine-threonine phosphatase interacting protein 1 (PSTPIP1) is an adaptor protein associated with the cytoskeleton that is mainly expressed in hematopoietic cells [1]. PSTPIP1 has an extended Fes-CIP4 homology domain, also known as F-BAR (where F refers to the Fer-CIP4 homology domain), at the N-terminal end [2,3], and an SH3 domain at the C-terminal end. Both domains have in common the ability to

Abbreviations
AP1, activator protein 1; CSK, C-terminal Src kinase; CTH, C-terminal homology domain; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-related kinase; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; HA, hemagglutinin; IL, interleukin; LYP, lymphoid phosphatase; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PAPA, pyogenic arthritis, pyoderma gangrenosum and acne; PBL, peripheral blood lymphocyte; PMA, 4β-phorbol 12-myristate 13-acetate; PSTPIP1, proline-serine-threonine phosphatase interacting protein 1; PTP-HSCF, protein tyrosine phosphatase hematopoietic stem cell fraction; shRNA, short hairpin RNA; TCR, T-cell receptor.

interact with poly-Pro motifs. Whereas the F-BAR domain binds to the C-terminal homology domain (CTH) poly-Pro motif of PEST phosphatases such as PTP-PEST [1] and to CD2 [4], the SH3 domain binds WASP [5], ABL [6] and CD2 [7] poly-Pro motifs. PSTPIP1 has been identified by yeast-two hybrid assays in two independent studies: in one case, protein tyrosine phosphatase hematopoietic stem cell fraction (PTP-HSCF, encoded by the gene *PTPN18*) was used as bait, and the protein was named PSTPIP1 [8]; and in the other case, the the cytoplasmic tail of CD2 was used as bait, and the the protein was named CD2-binding protein [7]. Mutations in PSTPIP1 cause the rare autoinflammatory syndrome called pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) [9]. In T cells, it has been proposed that PSTPIP1 negatively regulates CD2-dependent T-cell activation by recruiting the PEST phosphatase PTP-PEST [4,7,10,11]. Indeed, PTP-PEST has been associated with a negative regulatory role in T cells in overexpression studies [12,13]; however, a recent study, in which PTP-PEST was deleted in mouse T cells, showed that this phosphatase has a positive role in T-cell activation [14]. Nonetheless, it has also been reported that PSTPIP1 could facilitate the formation of the immune synapse by promoting actin polymerization, suggesting that PSTPIP1 can have a positive role in T-cell activation [4]. In addition, a mouse strain deficient in PSTPIP1 that does not show any gross abnormality has been generated [15]. Thus, as the role of PSTPIP1 in T cells is poorly understood, we initiated this study to deepen our knowledge on PSTPIP1 function in T cells, especially in relation to lymphoid phosphatase (LYP), a PEST tyrosine phosphatase that is associated with several autoimmune diseases. LYP has a single-nucleotide polymorphism that increases the risk to for several autoimmune diseases, such as arthritis and type I diabetes [16]. This polymorphism changes Arg620 to a Trp in the P1 Pro-rich motif of LYP, and reduces the binding to C-terminal Src kinase (CSK) [17], a kinase that works as negative regulator of SRC family kinases and consequently of antigen receptors in the immune system. In this study, we aimed to characterize this interaction and define the function of both proteins in T-cell activation. Our results show that PSTPIP1 is a negative regulator of T-cell receptor (TCR)-dependent T-cell activation, and not only of CD2-dependent T-cell activation. The interaction with PEST phosphatases does not seem to be required for this function. Instead, the mutation of the PSTPIP1 SH3 domain blocked the inhibitory function that PSTPIP1 has in T cells.

Results

PSTPIP1 inhibits CD3-dependent and CD28-dependent T-cell activation

Previous studies have shown that PSTPIP1 negatively regulates CD2-dependent T-cell activation in mouse T cells [7]. To verify this function of PSTPIP1 in human T cells, we tested whether PSTPIP1 regulated T-cell activation upon TCR and CD28 stimulation with and without CD2 costimulation. In these assays, PSTPIP1 was transfected in Jurkat T cells along with a plasmid that expresses luciferase under the control of the interleukin (IL)-2 minimal promoter. Induction of the IL-2 promoter was detected by luciferase assays upon stimulation with CD2, CD3 and CD28 antibodies. Transfection of PSTPIP1 inhibited T-cell activation not only after CD2 stimulation but also after CD3 and CD28 costimulation, or a combination of both (Fig. 1A). To further understand how proximally to the TCR PSTPIP1 performs its function, we assessed whether transient transfection of PSTPIP1 inhibited T cells activated by 4β-phorbol 12-myristate 13-acetate (PMA) and ionomycin. PSTPIP1 inhibited T-cell activation not only upon CD3 and CD28 costimulation, as before, but also upon CD3 and PMA costimulation, and upon PMA and ionomycin costimulation (Fig. 2B). We also tested whether the effect of PSTPIP1 on TCR signaling was dependent on the amount of PSTPIP1 transfected in Jurkat cells. Inhibition of TCR signaling increased proportionally with the amount of PSTPIP1 transfected (Fig. 1C). In contrast, T-cell activation was increased when PSTPIP1 expression was reduced with a specific short hairpin RNA (shRNA) (Fig. 1D), further supporting the inhibitory role of PSTPIP1 in TCR signaling. Altogether, these data show that PSTPIP1 inhibits T-cell activation upon CD3 and CD28 engagement, independently of CD2 costimulation. Furthermore, inhibition of T-cell activation by PMA and ionomycin indicates that PSTPIP1 regulates TCR signaling downstream of TCR proximal signaling events.

PSTPIP1 regulates several signaling pathways

Induction of IL-2 in T cells is regulated by several transcription factors activated upon TCR and CD28 engagement. Given that expression of PSTPIP1 regulated induction of the IL-2 minimal promoter, we next tested whether PSTPIP1 was able to regulate the activation of different transcription factors involved in IL-2 induction, such as nuclear factor of

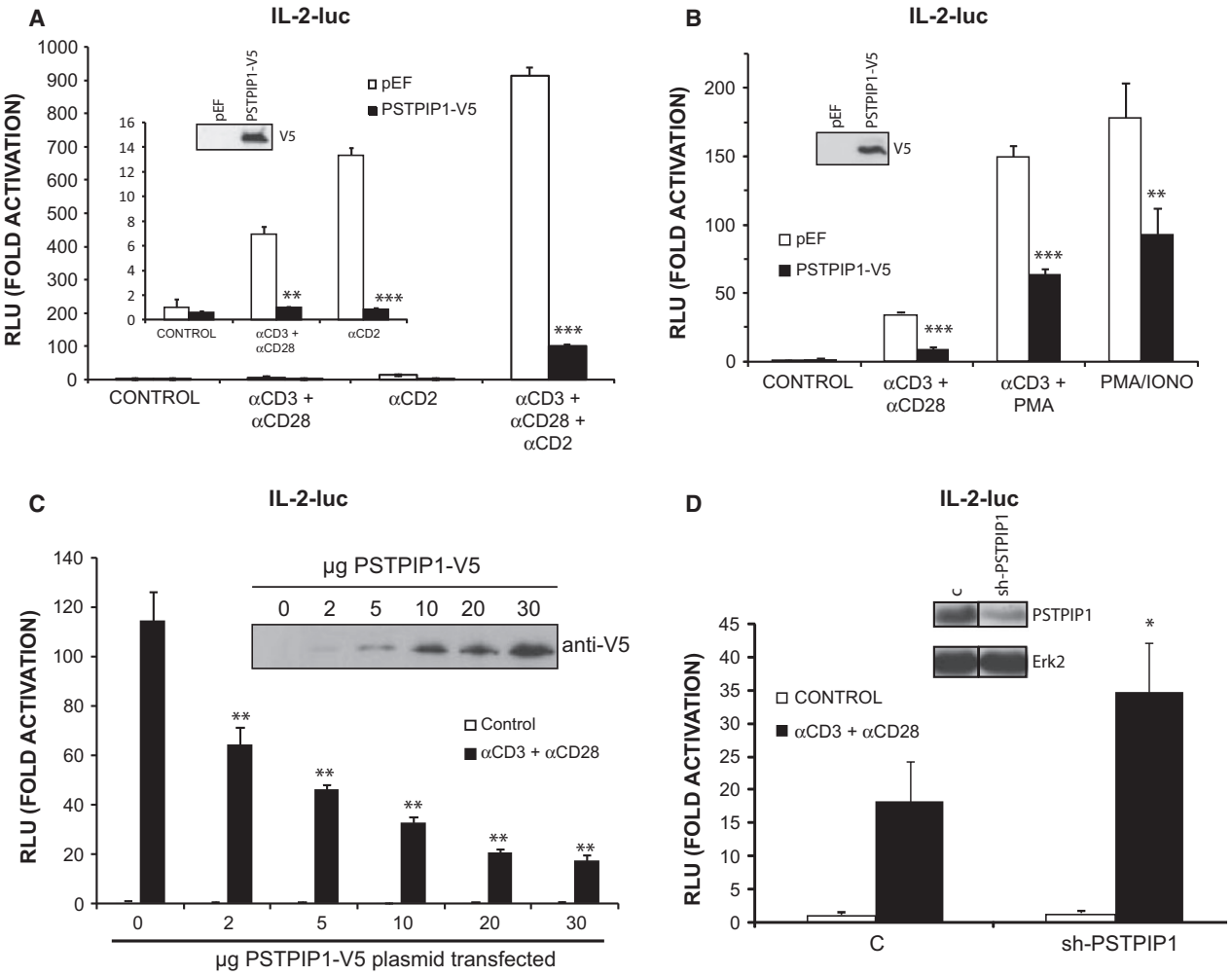

Fig. 1. PSTPIP1 inhibits TCR signaling downstream of the TCR. (A) Activation of a luciferase reporter gene driven by the IL-2 minimal promoter in Jurkat cells cotransfected with PSTPIP1-V5 and stimulated through the TCR, CD28 receptor, and CD2 receptor. The insert shows the expression of PSTPIP1-V5 detected by immunoblotting (IB). $^{**}P < 0.01$ and $^{***}P < 0.001$ for comparison of cells transfected with PSTPIP1-V5 and cells transfected with empty vector (pEF). (B) Activation of a luciferase reporter gene driven by the IL-2 minimal promoter in Jurkat cells cotransfected with PSTPIP1-V5 and stimulated with αCD3, αCD28, PMA, and ionomycin (IONO), as indicated. The insert shows the expression of PSTPIP1-V5 detected by IB. $^{**}P < 0.01$ and $^{***}P < 0.001$ for comparison of cells transfected with PSTPIP1-V5 and cells transfected with empty vector (pEF). (C) Dose response of PSTPIP1 for inhibition of the IL-2-luc reporter used in (A) and (B). The insert shows the expression of PSTPIP1-V5 detected by IB. $^{**}P < 0.01$ as compared with cells transfected with empty vector (pEF). (D) Activation of a luciferase reporter gene driven by the IL-2 minimal promoter in Jurkat cells transfected for 72 h with either control or PSTPIP1 suppression vector, and stimulated through the TCR and CD28 receptor. Expression of PSTPIP1 and ERK, as a loading control, was detected by IB. $^{*}P < 0.05$ as compared with cells transfected with a control shRNA (C).

activated T cells (NF-AT), activator protein 1 (AP1), and nuclear factor-κB (NF-κB). Activation of these transcription factors was detected with luciferase assays. In these experiments, expression of PSTPIP1 inhibited the activation of different transcription factors: NF-AT/AP1 (Fig. 2A), AP1 (Fig. 2B), Gal4-ELK (Fig. 2C), and NF-κB (Fig. 2D). These data indicate that PSTPIP1 regulates activation of different signaling pathways activated by the TCR and CD28 receptor.

The PSTPIP1–LYP interaction is mediated by the PSTPIP1 F-BAR domain and the LYP CTH motif

The inhibitory role of PSTPIP1 in T cells has been associated with its interaction with the PEST phosphatase PTP-PEST (PTPN12) [6,16]. As this group of phosphatases contains other two proteins, PTP-HSCF (PTPN18) and LYP (PTPN22), we initially determined the expression of these phosphatases and PSTPIP1 in Jurkat T cells and peripheral blood lymphocytes

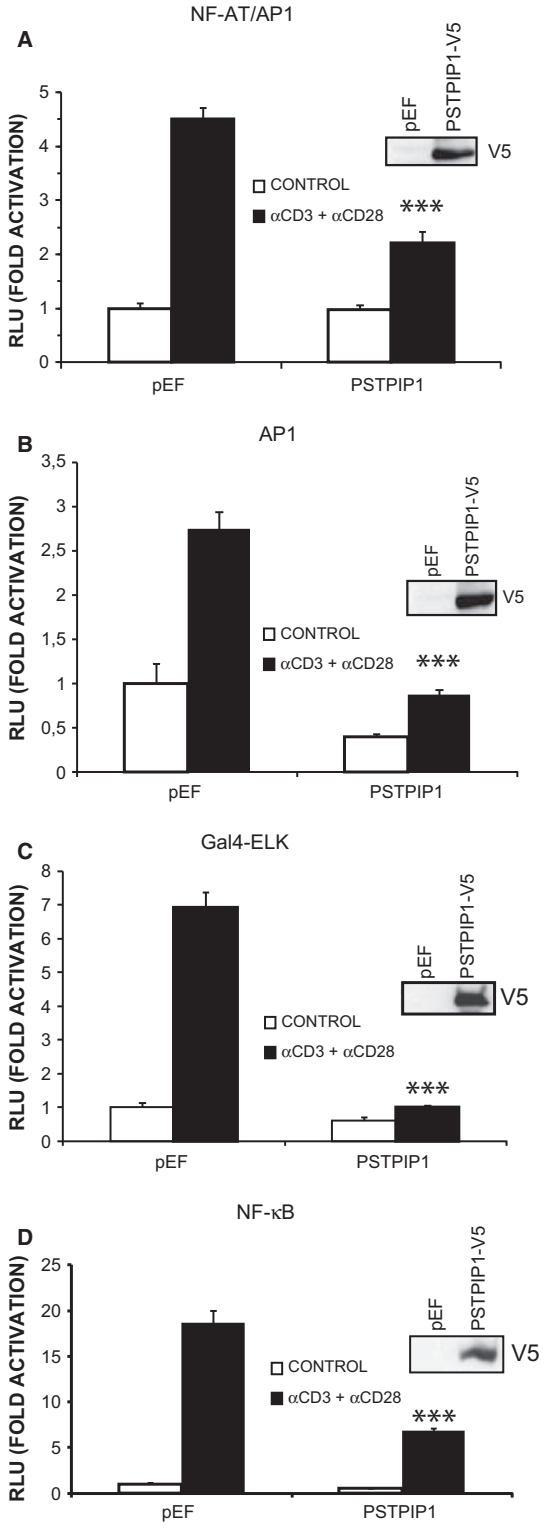

Fig. 2. Regulation of different transcription factors by PSTPIP1. Activation of a luciferase reporter gene driven by NF-AT/AP1 sites of the IL-2 promoter (A), AP1 (B), Gal4-ELK (C) or NF-κB (D) in Jurkat cells cotransfected with PSTPIP1-V5. The insert shows the expression of PSTPIP1-V5 as detected by western blotting in each assay. ***$P < 0.001$ as compared with cells transfected with pEF and stimulated through the TCR and CD28 receptor.

(PBLs). PSTPIP1 expression in Jurkat cells was hardly detectable by western blotting. To compare expression in PBLs and Jurkat cells, we scanned the bands and calculated the PSTPIP1/extracellular signal-related kinase (ERK)2 ratio, which showed that PSTPIP1 expression in Jurkat cells was five-fold lower than in PBLs (Fig. 3A). PTP-PEST was the only phosphatase of this family absent from Jurkat cells, in keeping with previous data [13]. Expression of LYP in Jurkat cells was higher than in PBLs, whereas expression of PTPN18 was lower. Then, we analyzed the PSTPIP1–LYP interaction. First, we examined whether PSTPIP1 could bind to LYP by coimmunoprecipitation. PSTPIP1 was coprecipitated with LYP in PBLs and Jurkat cells (Fig. 3B), in agreement with a recent report [18]. This interaction was not affected after T cell activation by pervanadate treatment (data not shown). Next, we determined how PSTPIP1 binds to LYP. To this end, we cloned the N-terminal F-BAR domain and the SH3 domain, as well as the full-length protein, with enhanced green fluorescent protein (EGFP). Immunoprecipitation assays of HEK293 cells transiently transfected with these PSTPIP1 constructs, along with full-length LYP, showed that PSTPIP1–LYP binding was mediated by the F-BAR domain (Fig. 3C). PTP-PEST bound in the same way to the PSTPIP1 F-BAR domain (data not shown), in agreement with data reported previously [11]. As the W232A mutation in the F-BAR domain of PSTPIP1 abrogates binding to PTP-PEST and PTP-HSCF [1,19], we tested the effect of this mutant on the LYP interaction. The W232A mutation also abolished LYP binding (Fig. 3D). In contrast, the W396A mutation, which changes a conserved residue in SH3 domains that is critical in ligand binding, did not impede the LYP interaction with PSTPIP1 (Fig. 3D). We followed the study of the PSTPIP1–LYP interaction by determining whether PSTPIP1 binds to the CTH motif of LYP, as has been reported for PTP-PEST and PTP-HSCF [1,8]. Thus, we deleted the CTH motif from LYP, and observed that this deletion abrogated the LYP–PSTPIP1 interaction (Fig. 3E). Additionally, in this experiment we tested whether the two variants of LYP, LYP-R and LYP-W, which are associated with several autoimmune diseases [20], bind to PSTPIP1, and no difference was observed (Fig. 3E). We also studied whether other LYP Pro-rich motifs could bind to PSTPIP1 in pulldown assays of Jurkat cell lysates, and concluded that the only Pro-rich

motif that bound to PSTPIP1 was the CTH motif (Fig. 3F). Altogether, these results show that LYP and PSTPIP1 form a stable complex in T cells. This complex is formed by the association of the PSTPIP1 F-BAR domain and the LYP CTH motif. It is also worth mentioning that PSTPIP1 binds similarly to both LYP isoforms (Arg620 and Trp620).

The inhibitory role of PSTPIP1 in TCR signaling depends on its SH3 domain

PSTPIP1 binds through the F-BAR domain to PEST phosphatases, which have been reported to negatively regulate TCR signaling [12,13]. We therefore tested whether the PSTPIP1 W232A mutation, which abrogates binding to PEST tyrosine phosphatases [19] (Fig. 3D), was still able to inhibit T-cell activation upon CD3 and CD28 costimulation. Expression of PSTPIP1-W232A in Jurkat cells showed a similar effect as that of wild-type PSTPIP1 on the activation of the IL-2 promoter in luciferase assays (Fig. 4A), in contrast to previous data reported for CD2-dependent T-cell activation in mouse T cells [10]. As PSTPIP1 binding to PEST phosphatases seemed to be dispensable for inhibition of TCR signaling, we tested the LYP-ΔCTH motif to verify whether this interaction was required by LYP to regulate TCR signaling. These assays showed that PSTPIP1 binding was not required by LYP to inhibit TCR signaling (Fig. 4B). We also coexpressed PSTPIP1-W232A along with LYP-ΔCTH to determine whether they could still cooperate in the negative regulation of TCR signaling. These mutant proteins were still able to cooperate in the regulation of TCR signaling, despite abolition of the LYP–PSTPIP1 interaction, (data not shown). As our data suggested that the PSTPIP1 F-BAR domain is not responsible for the negative regulatory role played by PSTPIP1 in T-cell activation, we reasoned that this function might be ascribed to its SH3 domain. We therefore transfected the W396A mutant, which abrogates binding to SH3 ligands, and tested whether this domain is involved in the negative regulation of T-cell activation by PSTPIP1. This mutation in the PSTPIP1 SH3 domain abolished the negative regulatory role played by PSTPIP1 in TCR signaling (Fig. 4C). Similar results were obtained when the SH3 domain was deleted from PSTPIP1 (data not shown), in keeping with previous data showing that a truncated version of PSTPIP1 does not inhibit CD69 induction upon CD2-dependent activation of mouse T cells [10]. However, the SH3 domain alone seemed to be not sufficient to carry out the function of PSTPIP1 in T cells when tested in Jurkat cells (Fig. 4D). Additionally, we tested whether PSTPIP1 and LYP regulated CD2-dependent T-cell activation in Jurkat cells. Our assays showed that, similarly to CD3 and CD28 T-cell activation, PSTPIP1 (Fig. 4E) and LYP (Fig. 4F) inhibited T-cell activation upon CD2 engagement, but, again, PSTPIP1 function seemed to be independent of LYP binding, as PSTPIP1 (Fig. 4E) and LYP-ΔCTH (Fig. 4F) with the W232A mutation inhibited T-cell activation in the same way as wild-type proteins. Taken together, these data show that PSTPIP1 negatively regulates CD2-dependent and CD2-independent T-cell activation, and that the PSTPIP1–LYP interaction is dispensable for this function, which is dependent on its SH3 domain.

PSTPIP1 mutants A230T and E250Q, which cause PAPA, differ in binding to LYP but do not affect PSTPIP1 regulation of T-cell activation

Two mutations in the PSTPIP1 F-BAR domain, A230T and E250Q, which are close to Trp232, which is critical for binding to PEST phosphatases, have been identified as the cause of the autoinflammatory disease known as PAPA [9]. To determine whether these mutations affect the role of PSTPIP1 in T-cell activation, we transfected these proteins along with wild-type PSTPIP1 into Jurkat cells and measured the induction of the IL-2 minimal promoter in luciferase assays after CD3 and CD28 costimulation. These mutations did not alter the inhibitory role played by PSTPIP1 in TCR signaling (Fig. 5A). To verify the interaction of these proteins with LYP, we used immunoprecipitation in Jurkat cells to test the binding of the A230T and E250Q mutants to LYP. This assay showed that, whereas the A230T mutant bound to LYP in the same way as wild-type PSTPIP1, the E250Q mutant showed no interaction (Fig. 5B). Similar results were obtained in HEK293 cells (data not shown). Collectively, these data indicate that mutants that cause the autoinflammatory syndrome called PAPA do not alter the regulatory role played by PSTPIP1 in T-cell activation. In addition, they support the notion that the LYP interaction is not critical for this function of PSTPIP1 in T cells.

Discussion

PSTPIP1 is an adaptor protein preferentially expressed in cells of the immune system that is involved in the regulation of T-cell activation, although its function is poorly defined. In contrast to previous reports that described an inhibitory role of PSTPIP1 restricted to CD2 T-cell activation in mouse T cells, our data show that PSTPIP1 also inhibits CD3 and CD28 T-cell activation without CD2 costimulation in human T cells. PSTPIP1 regulates the activation of several

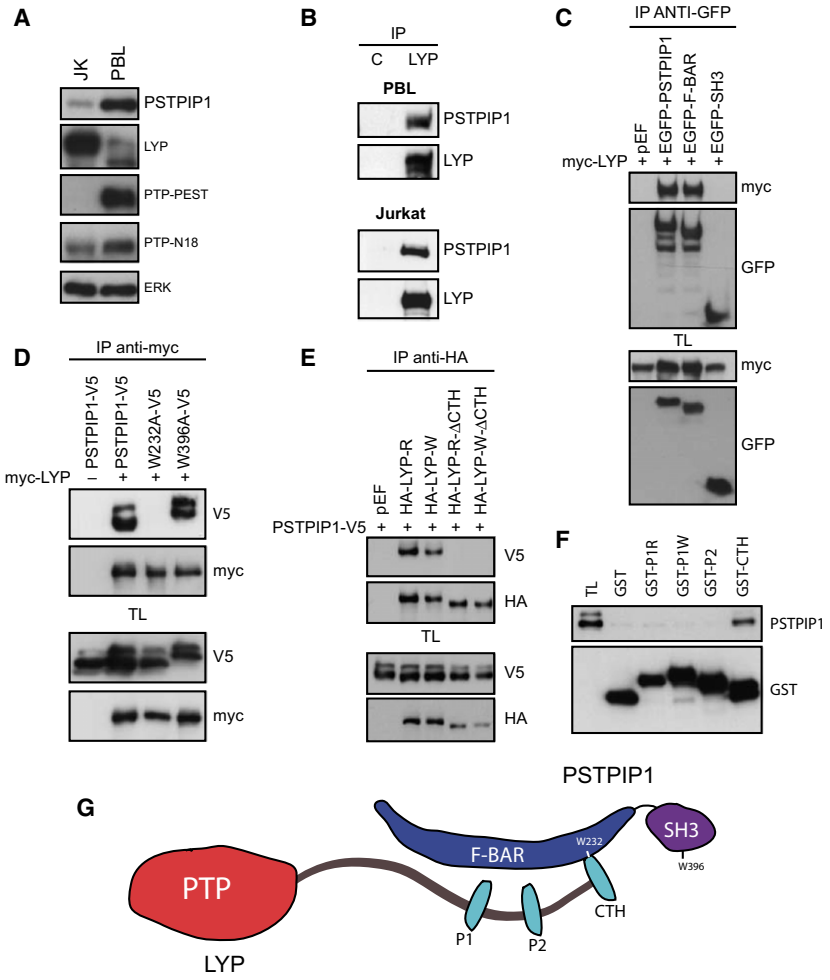

Fig. 3. Analysis of the PSTPIP1–LYP interaction. (A) Protein lysates (25 μg) of Jurkat cells and PBLs were subjected to SDS/PAGE, and, after transfer to nitrocellulose membranes, were blotted with the antibodies indicated on the right of the panel. (B) Lysates from PBLs and Jurkat cells were subjected to immunoprecipitation (IP) with antibody against LYP or IgG as control (C) and then blotted with antibody against PSTPIP1 (upper panels) to detect PSTPIP1. LYP was also blotted (lower panels) to verify the IP of this protein. (C) HEK293 cells were transfected with myc_3-LYP and PSTPIP1, F-BAR or SH3 domains fused to EGFP. Lysates were inmunoprecipitated with antibody against EGFP, and the interaction with PSTPIP1 was detected by immunoblotting (IB) with antibody against LYP. Expression of the proteins was verified by western blotting in total lysates (TL). (D) Interaction of myc_3-LYP with PSTPIP1-V5 mutants in the F-BAR domain (W232A) and SH3 domain (W396A) was verified by IB after LYP IP in transiently transfected HEK293 cells. (E) Total lysates (TL) of HEK293 cells transiently transfected with PSTPIP1-V5 and either HA-LYP-R620 or the autoimmunity risk isoform Trp620, as full length or with the CTH deleted (ΔCTH), or pEF as control, were subjected to IP and IB with the indicated antibodies. (F) Jurkat cell lysates were divided evenly and subjected to pulldown assays with the indicated Pro-rich motifs fused to GST. The presence of PSTPIP1 in the precipitates was visualized by IB with antibody against PSTPIP1, and GST–peptides were detected with an antibody against GST. TL show the presence of PSTPIP1 in the lysate. (G) Scheme representing the LYP–PSTPIP1 interaction and the structural elements of these proteins. LYP has a protein tyrosine phosphatase domain (PTP) in the N-terminus, followed by a central region called the interdomain and a C-terminal part that contains the Pro-rich motifs P1, P2, and CTH. PSTPIP1 contains an N-terminal F-BAR domain and an SH3 domain at the C-terminus. The positions of the residues mutated in PSTPIP1 in this study are indicated: Trp232 is located within the F-BAR domain, and Trp396 is located in the SH3 domain. As shown in this scheme, the LYP CTH motif binds to the F-BAR domain of PSTPIP1, where Trp232 is critical for this interaction. JK, Jurkat cells.

transcription factors, such as NF-AT, AP1, and NF-κB, that are relevant for immune cell function. We have studied LYP–PSTPIP1 binding, and our data show that this interaction is mediated by the F-BAR domain of PSTPIP1 and the CTH motif of LYP. The inhibitory role of PSTPIP1 in TCR signaling depends on the presence of an intact SH3 domain, in contrast to previous data showing that the inhibitory role of

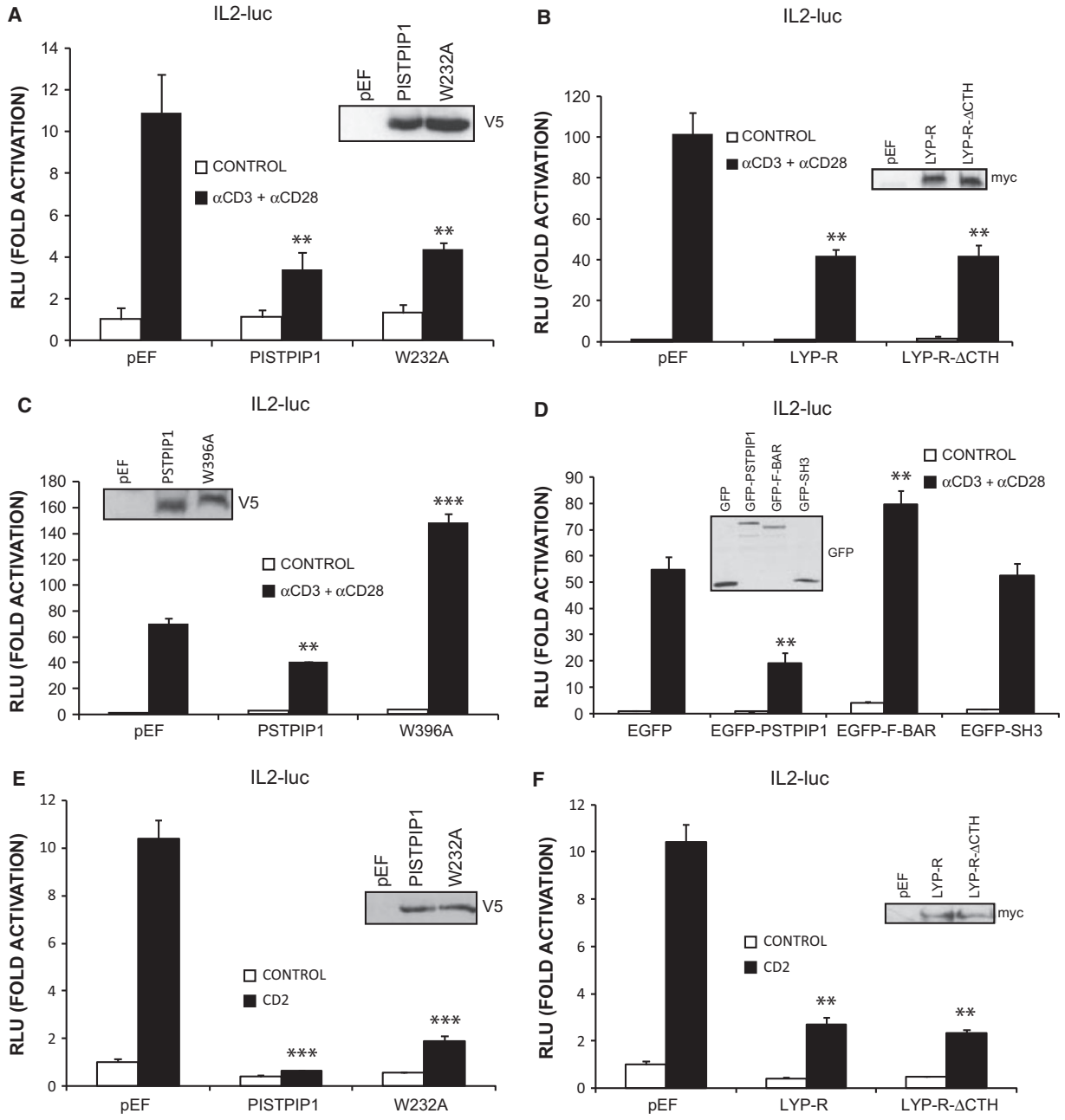

Fig. 4. The PSTPIP1 SH3 domain is critical for regulating T-cell activation. (A) Activation of a luciferase reporter gene driven by the IL-2 minimal promoter in Jurkat cells cotransfected with PSTPIP1-V5 and a mutant (W232A) that does not interact with LYP, and stimulated through the TCR and CD28 receptor. The insert shows the expression of PSTPIP1-V5 detected by immunoblotting (IB). (B) As in (A), a luciferase assay with LYP and a mutant (ΔCTH) that does not bind to PSTPIP1. The insert shows the expression of myc-LYP detected by IB. (C) Luciferase assay, as in (A) and (B), in Jurkat cells cotransfected with PSTPIP1-V5 and a mutant (W396A) that does not undergo SH3 ligand binding. The insert shows the expression of the different transfected proteins, visualized by IB. (D) As before, a luciferase assay with EGFP–PSTPIP1, and its domains separately, EGFP–F-BAR and EGFP–SH3. The insert shows the expression of the transfected proteins. (E, F) Luciferase assays as in (A) and (B), but with Jurkat cells were stimulated through the CD2 receptor. $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with stimulated cells transfected with empty vector (pEF).

PSTPIP1 in T cells was dependent on the PEST phosphatase interaction with the F-BAR domain. In addition, PSTPIP1s with mutations that cause the autoinflammatory disease PAPA, A230T and E250Q, regulate TCR signaling in a similar way to wild-type PSTPIP1, although they bind differently to LYP.

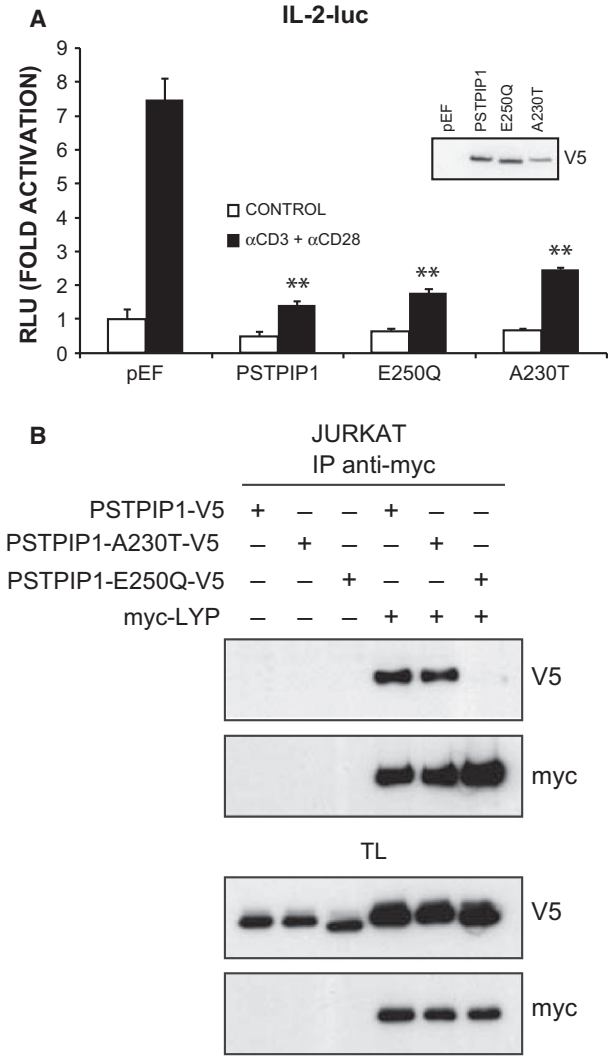

Fig. 5. PSTPIP1 mutants that cause the PAPA syndrome inhibit TCR signaling. (A) Activation of a luciferase reporter gene driven by the IL-2 minimal promoter in Jurkat cells cotransfected with PSTPIP1-V5, and the mutants that cause the PAPA autoinflammatory syndrome, PSTPIP1-E250Q-V5 and PSTPIP1-A230T-V5, as indicated. The insert shows the expression of PSTPIP1 proteins by immunoblotting (IB). $^{**}P < 0.01$ as compared with cells transfected with empty vector (pEF) and stimulated with antibodies against CD3 and CD28. (B) Jurkat cells were transfected with myc_3-LYP and PSTPIP1-V5, and the mutants that cause the PAPA syndrome PSTPIP1-E250Q-V5 and PSTPIP1-A230T-V5. Lysates were inmunoprecipitated with antibody against myc, and the presence of PSTPIP1 in the precipitates was detected by IB with antibody against V5. Expression of the transfected proteins was verified by western blotting in total lysates (TL). IP, immunoprecipitation.

Functional assays in Jurkat cells, which express five-fold less PSTPIP1 than PBLs, showed that expression of exogenous PSTPIP1 inhibited not only CD2-dependent but also CD2-independent T-cell activation through the TCR and CD28 receptor. In this respect, PSTPIP1 negatively regulated the IL-2 promoter in luciferase assays in a dose-dependent manner. Our data disagree with prior studies carried out in mouse T cells showing that PSTPIP1 only inhibits T-cell activation when CD2 is crosslinked alone or in conjunction with the TCR [7,10]. These discrepancies could be explained by the different models used, mouse T cells versus human T cells, as PSTPIP1 is overexpressed in both systems. Our data suggest that the CD2 interaction is not pivotal for the inhibitory role that PSTPIP1 plays in T-cell activation. It worth mentioning that reports on CD2–PSTPIP1 interaction disagree about the domain of PSTPIP1 that binds to the CD2 intracytoplasmic tail. One report suggests that the interaction is mediated by the PSTPIP1 SH3 domain [7], with a very low affinity (375 μM) [10], whereas another study found that PSTPIP1 binds through the coiled-coil region of the F-BAR domain [4], the same region used to bind to PEST phosphatases. Whether PTP-PEST binds at the same time as CD2 to PSTPIP1 has not been reported yet. PSTPIP1, like other members of the F-BAR family, binds to the membrane through phosphatidylinositol 4,5-bisphosphate [21], so binding to CD2 may recruit PSTPIP1 to the immune synapse, but our data suggest that this is not entirely necessary, as PSTPIP1 inhibits T-cell activation through the TCR without coligation of the CD2 molecule.

The interaction between PSTPIP1 and PEST phosphatases suggested that PSTPIP1 should function close to the initial events in TCR signaling; however, our data show that PSTPIP1 overexpression also inhibits the induction of the IL-2 promoter by PMA/ionomycin, which is considered to act further downstream in this pathway. These data, together with the fact that PSTPIP1 with the W232A mutation, which abrogates binding to PEST phosphatases, inhibits TCR signaling in the same way as the wild-type protein, support a model in which PSTPIP1 functions downstream of the early steps of the TCR signal transduction pathways.

The initial characterization of PSTPIP1 suggested that this protein could bind all of the PEST phosphatases [8], although direct binding of PSTPIP1 to LYP was only shown in a recent report [18]. Here, we have studied the PSTPIP1–LYP interaction, which has not been done before. Both LYP and PSTPIP1 are expressed in human lymphocytes. The three PEST phosphatases are present in PBLs, with LYP showing the lowest expression in both cell types. Interestingly, we detected the expression of PTPN18 in T cells, suggesting that its expression is not confined to

hematopoietic stem cells, as originally claimed [22]. Whether PSTPIP1 has a redundant or a specific role in complex with each phosphatase has not been fully clarified. In this respect, the presence of a polymorphism in LYP associated with several autoimmune diseases, and the dramatic effects of removing PTP-PEST from mice [23], indicate that these phosphatases have some specific and critical functions. PSTPIP1 binds to LYP in lymphocytes, and this interaction is not affected upon PV treatment. Similarly, the PTP-PEST interaction with PSTPIP1 was not affected by Tyr phosphorylation [1]. The PSTPIP1–LYP interaction is mediated by the LYP CTH motif and by the F-BAR domain of PSTPIP1, following the same model proposed for other PEST phosphatases [1,6,8]. Interestingly, it was possible to detect a complex formed by LYP, PSTPIP1 and CSK, a well-known ligand of LYP, indicating that binding of one of these proteins to LYP does not block the interaction with the other one (data not shown). However, the possible function of this complex remains unknown.

Although it has been proposed that inhibition of T-cell activation by PSTPIP1 is attributable to the interaction with PTP-PEST [10,11], our data show that wild-type PSTPIP1 regulates TCR signaling similarly to the W232A muntant, which does not bind to LYP or any other PEST phosphatase, supporting the idea that the inhibitory role of PSTPIP1 in T cells is independent of PEST phosphatases. In this regard, a recent report has shown that PTP-PEST has a stimulatory role in T-cell activation [14]. Similarly, deletion of the CTH motif in LYP did not change the inhibitory role of LYP in TCR-dependent or CD2-dependent T-cell activation. These findings regarding the CTH motif, along with our previous work [17], indicate that the Pro-rich motifs of LYP are not required for regulation of TCR signaling. As the F-BAR domain seems not to be required for regulation of T-cell activation, we studied the SH3 domain. Deletion of this domain or the W396A mutation in the PSTPIP1 SH3 domain, which abrogates ligand binding, blocked the inhibitory role of PSTPIP1 in T-cell activation. This effect is observed either upon CD2-dependent or CD3-dependent and CD28-dependent T-cell activation. However, the SH3 domain of PSTPIP1 is not sufficient to inhibit T-cell activation.

Analysis in T cells of the role of PSTPIP1 mutations that cause the autoinflammatory disease called PAPA, A230T and E250Q, showed no change in the inhibitory role that PSTPIP1 plays in these cells, indicating that PSTPIP1 function in T cells is not relevant for this disease. However, these mutations show a distinct role in the PSTPIP1–LYP interaction; whereas A230T does not affect this interaction, E250Q abolishes it, in contrast to data reported on the PTP-PEST–PSTPIP1 interaction, which showed that these mutations abrogate binding to PTP-PEST [9]. These data indicate that PSTPIP1 could bind to PTP-PEST and LYP differently. As the interaction between PSTPIP1 and PTP-PEST has been implicated in the mechanism underlying PAPA, analysis of the differences between the PTP-PEST and LYP interactions deserve further attention.

In conclusion, in this study we have shown that PSTPIP1 regulates T-cell activation upon CD3 and CD28 stimulation, independently of CD2 costimulation. PSTPIP1 acts downstream of proximal TCR signaling, inhibiting several transcription factors. Regulation of T-cell activation by PSTPIP1 depends on its SH3 domain, probably because of the interaction with some SH3 ligand, a possibility that we are currently investigating.

Experimental procedures

Antibodies and reagents

Reagents for cell culture were from Lonza (Verviers, Belgium). The antibodies used in this work were: anti-hemagglutinin (HA) Ig (mouse monoclonal IgG1) from Covance (Berkely, CA, USA); anti-glutathione-S-transferase (GST) Ig (mouse monoclonal, B-14), anti-myc Ig (mouse monoclonal, 9E10) and anti-ERK2 Ig (rabbit polyclonal, C14) from Santa Cruz Biotechnology (CA, USA); anti-V5 Ig (mouse monoclonal IgG2a) from Invitro-gen (Carlsbad, CA, USA); anti-ABL Ig (mouse monoclonal IgG1), anti-CD2/CD2R Ig (mouse monoclonal), anti-CD3 Ig (mouse monoclonal IgG1, UCHT1) and anti-CD28 Ig (mouse monoclonal IgG1, CD28.2) from BD Pharmingen (Franklin Lakes, CA, USA); anti-LYP Ig (goat polyclonal, AF3428) from R&D Systems (Minneapolis, MN, USA); rabbit polyclonal anti-PTP-PEST Ig (A301-300A) from Bethyl (Montgomery,TX, USA); rabbit polyclonal anti-PTP-N18 Ig (8311) from Cell Signaling Biolabs (Danvers, MA USA); and anti-green fluorescent protein (GFP) rabbit polyclonal Ig from eBioscience (San Diego, CA, USA). The rabbit polyclonal PSTPIP1 antibody was generated against the whole protein by Biomedal (Sevilla, Spain).

Plasmids and mutagenesis

The PSTPIP1 wild-type sequence in pcDNA3.1/V5-His and the mutants A230T and E250Q were kind gifts from C.A. Wise (Sarah M. and Charles E. Seay Center for Musculoskeletal Research, Scottish Rite Hospital for Children, Dallas, TX, USA), and ABL was a kind gift from S. P. Goff (Department of Biochemistry and Molecular Biophysics,

Columbia University, New York, NY, USA). LYP was cloned in the *Bam*HI and *Not*I sites of pEF1/V5-HisA (Invitrogen), to which a myc_3 tag was added at the N-terminus between the *Kpn*I and *Bam*HI sites of this vector. Standard protocols were used to generate the plasmids employed in the present work. Point mutations were introduced with the QuickChange Mutagenesis Kit (Agilent, CA, USA), according to the manufacturer's recommendations. The plasmids generated in this study were verified by DNA sequencing. To knock down the expression of PSTPIP1, a 29mer shRNA (CATACAGCCGTCCTGCGGCATGAT AAAGA) construct in the pRFP-C-RS plasmid against PSTPIP1 was used (Origene, Rockville, MD, USA).

Cell culture and transfections

HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units·mL^{-1} penicillin G, and 100 μg·mL^{-1} streptomycin. HEK293 cells were transfected by use of the calcium phosphate precipitation method [24]. Jurkat T cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units·mL^{-1} penicillin G, and 100 μg·mL^{-1} streptomycin. Jurkat T cells were transfected by electroporation, as described previously [25]. PBLs were purified by centrifugation at 700 ***g*** for 30 min on cushions of Ficoll-Hypaque (GE Healthcare, Little Chalfont, UK) from healthy donor buffy coats obtained from the regional blood bank with approval of its ethics committee. Monocytes/macrophages were removed by adherence to plastic overnight at 37 °C.

Immunoprecipitation, GST pulldown, SDS/PAGE, and immunoblotting

These techniques were carried out as reported previously [17,25].

Luciferase assays

Luciferase activity in Jurkat cells was measured as described previously [25,26]. Briefly, 20×10^6 Jurkat cells were electroporated with 20 μg of empty pEF vector or the indicated plasmids, as well as 3 μg of different luciferase reporters and 0.5 μg of a *Renilla* luciferase reporter, which was used for normalization. Cells were stimulated with antibodies against CD3 and CD28 receptors 24 h after transfection for at least 6 h. Then, cells were lysed, and the lysates clarified by centrifugation at 16000 ***g*** for 10 min were used to measure the luciferase activity with the Dual Luciferase system (Promega), according to the manufacturer's recommendations. The statistical significance of the data was determined with Student's *t*-test, and is indicated for each figure.

Acknowledgements

The authors thank E. González for her technical assistance and the staff of Centro de Hemoterapia y Hemodonación de Castilla y León for their help with the preparation of leukocytes. We are also grateful to C. Wise and S. P. Goff for reagents. This work was supported by a grant (SAF2009-09724) from the Ministerio de Ciencia e Innovación (MICINN), and by the Fundación Ramón Areces.

Author contributions

A. Alonso, Y. Bayón, T. Marcos, V. Ruiz-Martín, and M. Luisa de la Puerta: planned experiments. A. Alonso, Y. Bayón, T. Marcos, V. Ruiz-Martín, M. Luisa de la Puerta, M. del Carmen Rodríguez, and A. G. Trinidad: performed experiments. A. Alonso, Y. Bayón, T. Marcos, and V. Ruiz-Martín: analyzed data. M. Sánchez Crespo, Y. Bayón, and A. Alonso: wrote the paper. M. Angel de la Fuente: discussed results and assisted in manuscript writing.

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