FEBS 28738 FEBS Letters 574 (2004) 37–41

PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3ζ signalosome and downstream signaling to PKCθ

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Received 23 May 2004; revised 27 July 2004; accepted 30 July 2004

Available online 13 August 2004

Edited by Beat Imhof

Abstract Engagement of the immunoinhibitory receptor, programmed death-1 (PD-1) attenuates T-cell receptor (TCR)-mediated activation of IL-2 production and T-cell proliferation. Here, we demonstrate that PD-1 modulation of T-cell function involves inhibition of TCR-mediated phosphorylation of ZAP70 and association with CD3ζ. In addition, PD-1 signaling attenuates PKCθ activation loop phosphorylation in a cognate TCR signal. PKCθ has been shown to be required for T-cell IL-2 production. A phosphorylated PD-1 peptide, corresponding to the C-terminal immunoreceptor tyrosine-switch motif (ITSM), acts as a docking site in vitro for both SHP-2 and SHP-1, while the phosphorylated peptide containing the N-terminal PD-1 immunoreceptor tyrosine based inhibitory motif (ITIM) associates only with SHP-2.

Keywords: TCR signaling; CD3ζ ITAM; ZAP70; Immunoinhibition

1. Introduction

The immunoreceptor, programmed death-1 (PD-1), is a member of the immunoglobulin superfamily that is expressed on a subset of thymocytes and on activated T- and B-cells, and monocytes [1–3]. Mice deficient in PD-1 display multiple autoimmune defects and a loss of peripheral tolerance [4–7]. The ligands for PD-1, PD-L1 and PD-L2, are members of the B7 co-stimulatory molecules family [8] and are expressed on antigen presenting cells, endothelial and epithelial cells, and on activated lymphocytes [9–12]. Interaction of PD-1 with the ligands, PD-L1 and PD-L2, inhibits co-stimulation mediated proliferation and cytokine secretion in T-cells [9,13,14]. The inhibitory activity of PD-L1 and PD-L2 requires the expression of PD-1, as PD-1 deficient T-cells show no inhibition of anti-CD3 mediated proliferation by PD-1 ligands [9,13]. PD-1 acts as an immunoinhibitory molecule by downmodulating both T-cell and B-cell responses [6,8,15].

PD-1 cytoplasmic region contains an N-terminal sequence VD**Y**GEL and a C-terminal immunoreceptor tyrosine-switch motif (ITSM) TE**Y**ATIV [16], which in addition to PD-1 has been described in CD2 subfamily members, as well as, cytoplasmic domains of the members of the SHP-2 substrate 1, sialic acid-binding Ig-like lectin, carcinoembryonic Ag, and leukocyte-inhibitory receptor families. The PD-1 ITSM associates with SHP-2 and is required for PD-1 signaling in B cells [8,17]. The N-terminal sequence is similar to the sequence I/L/VX**Y**XXL/V, which is defined as the immunoreceptor tyrosine-based inhibitory motif (ITIM) that recruits src homology-2 (SH2) domain containing phosphatases [18]. In B-cells, engagement of PD-1 inhibits B-cell receptor (BCR) mediated Ca^{2+} mobilization and phosphorylation of key signaling molecules including Igβ, Syk, PLCγ2 and ERK1/2 [17]. The inhibition of the BCR signaling cascade has been shown to be directed by the recruitment of the SH2-domain containing tyrosine phosphatase 2 (SHP-2) to the C-terminal phosphotyrosine of PD-1, but not the ITIM phospho-tyrosine [17].

We report here some molecular events in the PD-1 mediated downmodulation of T-cell receptor (TCR) signaling. We present evidence to show that in addition to the ITSM PD-1 peptide, the ITIM phospho-tyrosine peptide can also serve as a docking site for SHP-2. We propose a membrane proximal inhibitory mechanism for PD-1 in attenuation of ZAP70/CD3ζ signalosome phosphorylation and consequently downmodulation of the TCR signaling pathway.

2. Materials and methods

2.1. Cells and stimulations

Mononuclear cells (Biological Specialties, Colmar, PA) were layered on Ficoll–Histopaque (stem cell technologies) and the buffy coat was collected following centrifugation. T-cell blasts were generated after 4 days in PHA (0.001%) alone and 3 days in PHA and 2 ng/ml IL-2. Jurkat cells expressing hPD-1 with IRES-GFP were constructed using an MSCV2.2-derived retroviral vector (provided by Dr. Kenneth Murphy, Washington University, St. Louis, MO). Virus-containing supernatants were produced in the 293-VSVg packaging line and used to spin-infect cells followed by sorting for GFP expression. Cell surface PD-1 expression was confirmed by FACS analysis (data not shown) and from several experiments determined to be comparable to PD-1 expression on PHA/IL-2 stimulated T-cell blasts. Recombinant hPD-1.Fc and hPD-L1.Fc were generated as previously described [13]. Cells were stimulated with anti-CD3ε and IgG2a, or anti-CD3ε and

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0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.07.083

hPD-L1.Fc, in the presence of soluble anti-CD28 by immobilizing on tosyl-activated beads (Dynal). Culture supernate was harvested for IL-2 ELISA (R&D systems).

2.2. Biochemical and Western blot analysis

Whole cell lysates were prepared in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, or 50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, with protease inhibitors (Boehringer–Mannheim) and phosphatase inhibitors (Calbiochem). Protein concentration was determined (Pierce colorimetric assay) and extracts subjected to SDS–PAGE and analyzed by Western blots; anti-CD3ζ (Immunotech), anti-pTyr (Upstate), ERK1/2, and phospho-PKCθ T_{538} (Cell Signaling Technology), PKCθ, pERK1/2, and SHP-2 (Santa Cruz), SHIP, SHP-1 (Transduction Labs), IgG2a, anti-CD3ε, anti-murine IgG, and anti-CD28 (BD Biosciences), anti-PD-1 (Dr. T. Honjo, Kyoto University). In some cases, densitometric scans of bands were obtained to determine the extent of PD-1 mediated signal inhibition.

2.3. Peptide precipitation

Biotinylated peptides for precipitation (Research Genetics) – ITIM peptides: unphosphorylated (Y1); phosphorylated (pY1); scrambled (pY1mix); tyrosine to phenylalanine mutant (Y1-F), and ITSM peptides with cysteine to serine substitution (to eliminate potential peptide dimerization) at position –5 aa relative to the TE**Y**ATI motif: unphosphorylated (Y2); phosphorylated (pY2); scrambled (pY2mix); tyrosine to phenylalanine mutant (Y2-F). Jurkat cells ($\sim 50 \times 10^6$) treated for 10 minutes at 37 °C with PMA (10 ng/ml) and ionomycin (500 ng/ml) were lysed in modified RIPA buffer on ice. Lysates were precleared with streptavidin sepharose, followed by precipitation using peptides for 2 h and streptavidin sepharose for 1 h. Precipitations were analyzed by SDS–PAGE and probed for SHIP, or SHP-1, or SHP-2. For mass spectrometry analysis, SDS-PAGE bands were excised and subjected to in gel tryptic digestion.

3. Results and discussion

3.1. PD-1 engagement inhibits distinct TCR signal transduction pathways

We investigated the consequence of PD-1 engagement on TCR signaling pathways. TCR signaling induces distinct downstream signal transduction pathways, including ERK phosphorylation [19]. As in the case of B cell signaling, we observe significant PD-1 mediated inhibition of ERK activation (80% inhibition) in the presence of the TCR signal occurring as early as 2 min following PD-1 engagement (Fig. 1A). We verified PD-1 inhibition by monitoring IL-2 production in response to TCR activation using anti-CD3ε and either IgG_{2a} or PD-L.Fc coated beads (Fig. 1B).

Thereafter, we investigated a distinct effector arm of the TCR signaling cascade involving the novel PKC subfamily member, PKCθ, which is required for T-cell IL-2 production [20,21]. Phosphorylation at amino acid residue T_{538} in the PKCθ activation loop has been shown to support the catalytically active conformation of the enzyme [22]. Using a PKCθ T_{538} phospho-specific antibody, we show that activation of T-cells induces T_{538} phosphorylation within 2 min of stimulation (Fig. 1C). However, when TCR is co-ligated with PD-L1.Fc, PKCθ T_{538} phosphorylation signal is significantly inhibited, in excess of 90% inhibition of the T-cell activation signal, as detected by densitometric scanning of the results shown in Fig. 1C, indicating that PD-1 engagement downmodulates PKCθ activation signal. These results suggest that distinct arms of the TCR signal transduction pathway are inhibited by PD-1 engagement, with modulation in the signaling cascade upstream at a TCR proximal step.

3.2. PD-1 engagement inhibits the TCR phosphorylation signal

We next examined ZAP70 and CD3ζ tyrosine phosphorylation, a membrane proximal event in TCR signaling [19]. PD-1 mediated inhibition of IL-2 production in the PD-1 transduced Jurkat cell system was established using anti-CD3/IgG or anti-CD3/PD-L1 co-immobilized beads both in the absence and presence of soluble anti-CD28 (data not shown). Jurkat cells were stimulated for 2 minutes and analyzed by ZAP70 immunoprecipitation and Western blot using an anti-phospho-tyrosine antibody (Fig. 2A). TCR crosslinking results in ZAP70 tyrosine phosphorylation as shown in Fig. 2A left panel using anti phospho-tyrosine blot and right panel showing the loading control using anti ZAP70 blot. TCR induced ZAP70 phospho-tyrosine signal is inhibited within 2 min of co-

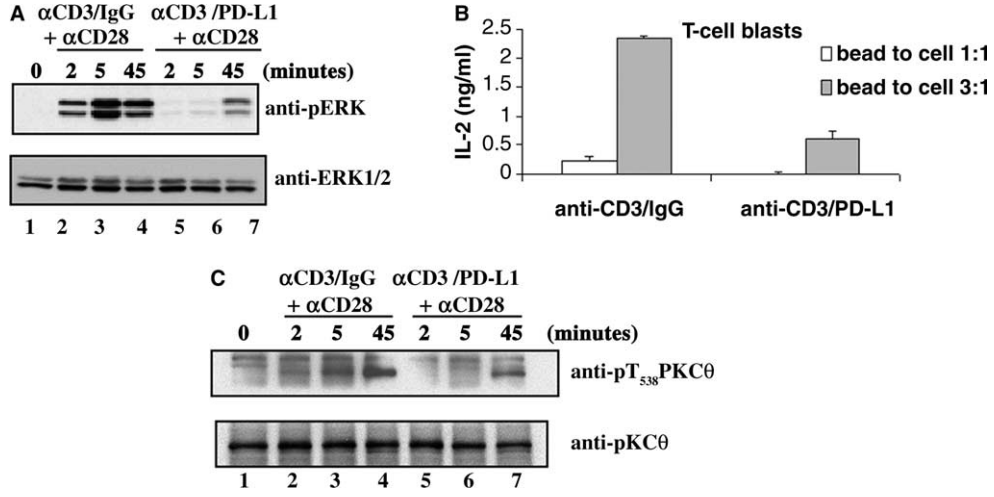


Fig. 1. PD-1 engagement inhibits PKCθ and ERK activation in TCR signal transduction. (A) Human T-cell blasts were stimulated with tosyl-activated beads co-ligated with anti-CD3ε (1 μg/10^7 beads) and either isotype control or hPD-L1.Fc (4 μg/10^7 beads) and anti-CD28 (5 μg/ml), for the indicated times. Cell lysates were analyzed by Western blots – first with a phospho ERK1/2 specific antibody followed with ERK1/2 antibody. (B) Human T-cell blasts were stimulated with tosyl-activated beads as in panel A at the indicated bead:cell ratios. IL-2 production was analyzed after 3 days in culture. (C) Human T-cell blasts were stimulated with tosyl-activated beads as in panels A and B for the indicated times, and cell lysates were analyzed by Western blots – probed first with a phosphoT_{538} PKCθ specific antibody followed with anti-PKCθ E7.

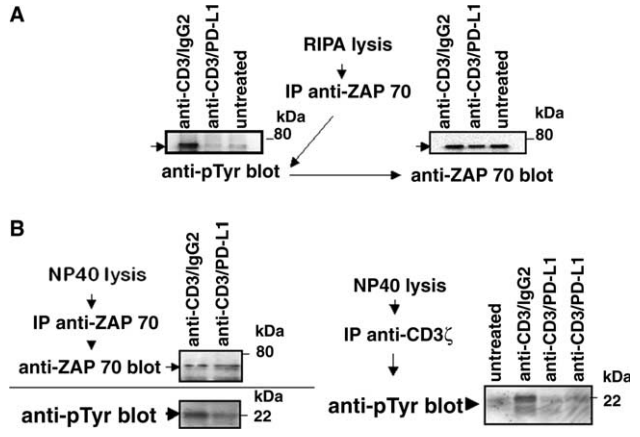


Fig. 2. PD-1 engagement inhibits ZAP70 and CD3ζ phosphorylation and association. (A) Jurkat cells were stimulated with tosyl-activated beads co-ligated with anti-CD3 (1 μg/10^7 beads) and either IgG isotype control or hPD-L1.Fc (4 μg/10^7 beads) for 2 min, and cells were lysed in RIPA lysis buffer. ZAP70 CD3ζ was immunoprecipitated from equal amounts of protein and immune complexes were analyzed by Western blot for anti phosphor-tyrosine and anti-ZAP70. (B) Jurkat cells were stimulated, then lysed in NP-40 lysis buffer to maintain protein interactions, followed by ZAP70 or CD3ζ immunoprecipitation from equal amounts of protein. Immunocomplexes were analyzed for anti phospho-tyrosine and anti-ZAP70.

ligation with PD-1 as shown in Fig. 2A. The extent of inhibition observed in these experiments as detected by densitometric scanning is ~85%.

Activated ZAP70 associates with tyrosine phosphorylated CD3ζ to potentiate the downstream TCR signal. [19,23]. Upon TCR crosslinking phosphorylated CD3ζ is detected as a 23 kD phospho-tyrosine protein in ZAP70 immuneprecipitated complex from Jurkat cell lysates, as shown in Fig. 2B left panels by the anti ZAP70 and antiphospho-tyrosine blots. In this experiment, we demonstrate that PD-1 engagement attenuates the TCR induced association of the 23 kD phospho-tyrosine protein with ZAP70 by about 50% as detected by densitometric scanning of the two lanes in Fig. 2B lower left panel. We further analyzed immunoprecipitated CD3ζ from cell lysates by anti phospho-tyrosine blots to show that TCR induced CD3ζ phospho-tyrosine signal is inhibited ~70%, upon TCR/PD-1 co-ligation (Fig. 2B right panel). This result shown is representative of four independent experiments which were repeated using equivalent amount of protein lysate in immuneprecipitations, as the anti CD3ζ antibody did not give adequate Western blot signal perhaps due to low affinity of the antibody in both the immunoprecipitation and blotting (actin Western blot controls not shown). Collectively, these findings indicate that the PD-1 signal attenuates TCR induced CD3ζ chain and ZAP70 tyrosine phosphorylation.

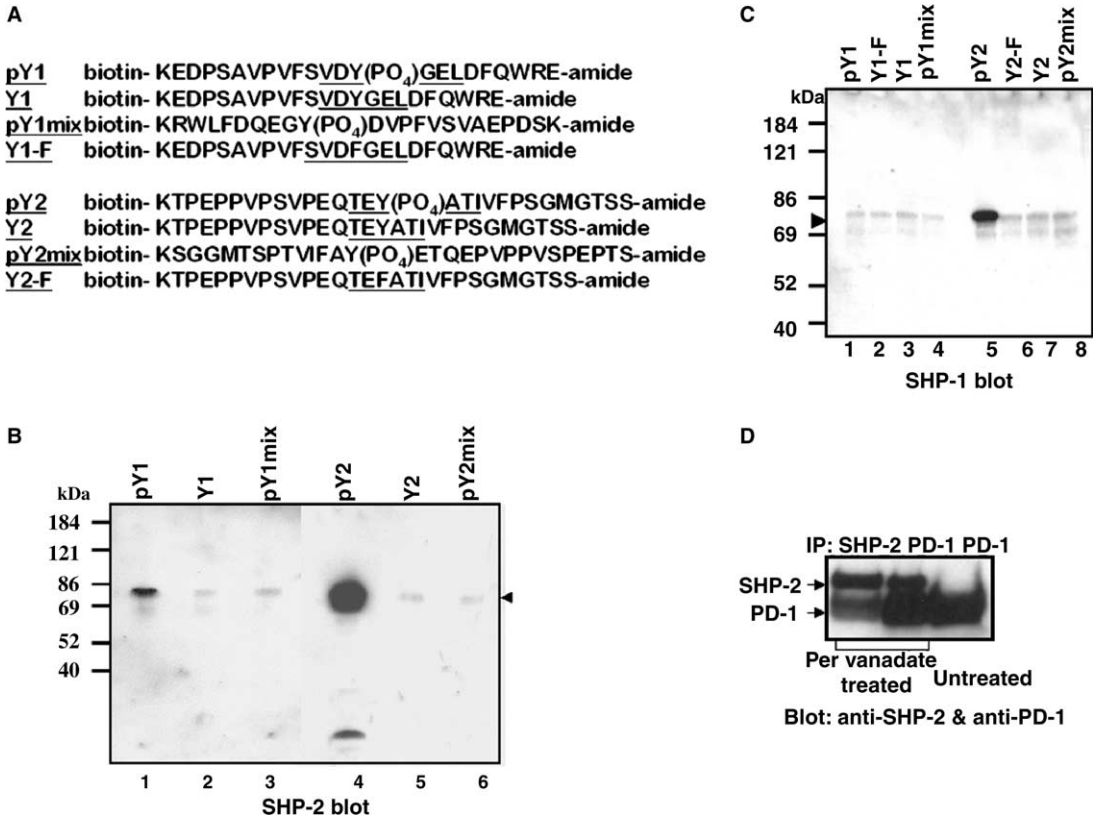


Fig. 3. Both SHP-1 and SHP-2 associate with PD-1 in a phosphorylation dependent manner. (A) Biotinylated peptides corresponding to the PD-1 ITIM (Y1) and ITSM (Y2) sequences used for precipitating proteins from Jurkat cell lysates. (B) Precipitated proteins from activated Jurkat cells as in panel B were analyzed for SHP-2 by Western blot. (C) Precipitated proteins from activated Jurkat cell lysates were analyzed for SHP-1 by Western blot. (D) Jurkat cells were either pervanadate treated or untreated and cells were lysed in NP-40 lysis buffer for SHP-2 and PD-1 immunoprecipitations. The immune complexes were analyzed by Western blot for both SHP-2 and PD-1.

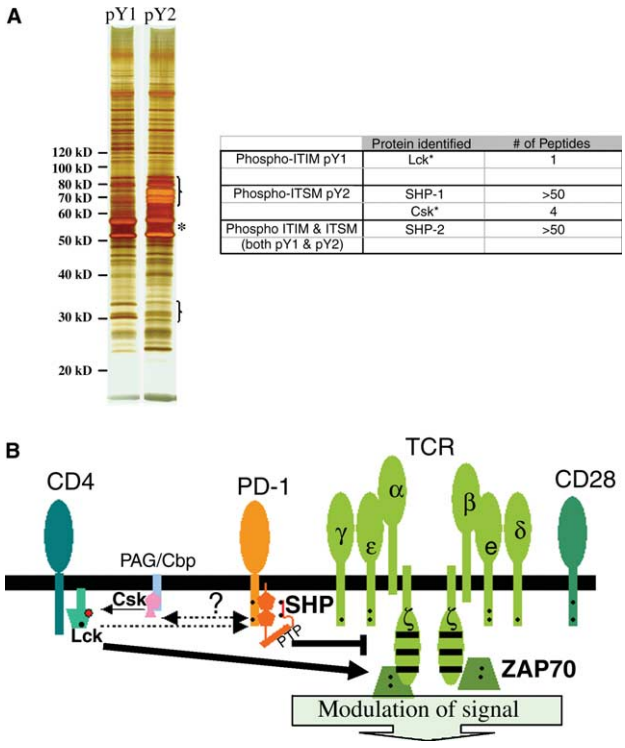
3.3. PD-1 recruits both SH2-domain tyrosine phosphatases, SHP-1 and SHP-2, in T-cells

In B cells, the PD-1 C-terminal ITSM has been shown to associate with SHP-2, and not SHP-1, and the N-terminal ITIM is not required [17]. We have shown previously SHP-2 phosphorylation upon PD-1 engagement in T-cells [9]. Therefore, we queried the PD-1:SHP-2 interaction of T-cells and asked if one or both of the cytoplasmic tyrosine motifs facilitate this interaction. We utilized a precipitation approach of activated Jurkat cell lysates with peptides corresponding to either the PD-1 ITIM or the PD-1 ITSM (Fig. 3A). Interestingly, SHP-2 associates with both the phospho-ITIM pY1 and phospho-ITSM pY2 peptides, however, a quantitatively higher amount of SHP-2 associates with phospho-ITSM pY2 than with phospho-ITIM pY1 (Fig. 3B, lanes 1 and 4). No SHIP binding to PD-1 peptides was detected in these studies (data not shown). SHP-1 binding is detected only in the phospho-ITSM pY2 peptide precipitated complex (Fig. 3C, lane 5). This suggests a phosphorylation dependent docking of SHP-1 specifically to the PD-1 phospho-ITSM pY2 and not the phospho-ITIM pY1 sequence.

To confirm a phosphorylation dependent PD-1:SHP-2 endogenous association, PD-1 was immunoprecipitated from pervanadate treated or untreated Jurkat cells and analyzed for co-precipitation (Fig. 3D). Treatment with pervanadate, an inhibitor of tyrosine phosphatases, will favor the detection of a tyrosine phosphorylation dependent interaction. SHP-2 and PD-1 co-precipitated from pervanadate treated cells and not untreated cells, consistent with a phosphorylation dependent association. We did not observe any endogenous PD-1:SHP-1 association (data not shown), perhaps due to differences in expression levels or a competing PD-1:SHP-2 association.

SHP-1 and SHP-2 association with PD-1 phospho-peptides was further confirmed by mass spectrometric identification of phospho-peptide precipitated protein bands. Fig. 4A shows the silver stained gel, indicating the major protein bands that resulted in the identified peptides as shown in the table. No endogenous PD-1:SHP-1 association was detected from pervanadate treated cells, and it is further likely that the homology between SHP-1 and SHP-2 led to the in vitro association observed with the phospho-ITSM pY2 peptide (Fig. 3). In excess of fifty peptides, each from SHP-1 and SHP-2 was detected in the peptide precipitated proteins identified by MS, again with SHP-1 being ITSM pY2 specific (Fig. 4A). Four C-terminal Src kinase (Csk) peptides were identified as ITSM pY2 specific and one Lck peptide as ITIM pY1 specific. Upon co-ligation of PD-1 and the TCR, the rapid phosphorylation of SH-2 domain phosphatase SHP-2 [9], and perhaps PD-1, may be mediated by a Src family kinase, thereby activating the phosphatase [24] and downmodulating the TCR signal (Fig. 4B). Consistent with this model Okazaki et al. [17] report that Src family kinase, Lyn, phosphorylates PD-1 in B-cells. In the TCR resting state, the negative regulatory tyrosine on Lck is phosphorylated by Csk [19,25]. Downmodulation of CD3ζ by PD-1 likely involves both tyrosine phosphorylation of SHP-2 and PD-1 and perhaps negative regulation of tyrosine kinase activities via Csk and Lck.

Crystal structure and mutation analysis of SHP-2 support a phosphatase active conformation when the SH-2 domains bind a biphosphorylated ligand [18,26,27]. We show that SHP-2 associates with both the ITIM pY1 and ITSM pY2 (Fig. 3B), suggesting that the in vivo association in T cells may involve binding to a biphosphorylated PD-1 cytoplasmic region. Lu et al. [24] report that phosphorylation of the two C-terminal tyrosine residues of SHP-2 significantly stimulates catalytic activity, again likely due to association of the biphosphorylated C-terminal tail with the SH2-domains. PD-1 engagement mediated phosphorylation of SHP-2 [9] may positively regulate phosphatase activity by a similar structural mechanism. The immunoinhibitory receptor BTLA (B and T lymphocyte attenuator), like CTLA-4, has homology to PD-1 [28]. SHP-2 can associate with the cytoplasmic tail of CTLA-4 and this association also requires phosphorylation of the tail motif by Lck [29]. Similarly, BTLA has been shown to recruit SHP-1 and SHP-2 upon crosslinking [28]. It is likely that BTLA engagement also results in SHP-1/2 being phosphorylated, as has been shown for SHP-2 by PD-1 ligation [9].



	Protein identified	# of Peptides
Phospho-ITIM pY1	Lck*	1
Phospho-ITSM pY2	SHP-1	>50
	Csk*	4
Phospho ITIM & ITSM (both pY1 & pY2)	SHP-2	>50

Fig. 4. PD-1 peptide precipitated protein identification and model for TCR downmodulation by PD-1. (A) Precipitated proteins from Jurkat cell lysates using the phospho-ITIM and phospho-ITSM peptides described in Fig. 3 were separated by SDS–PAGE and silver stained, followed by MS identification of in-gel tryptic digested peptides from sliced gel bands indicated by brackets (denoting the gel bands corresponding to SHP-1 and SHP-2 peptides) and asterix (denoting the gel band corresponding to Lck and Csk peptides). The table lists the peptide numbers identified for each protein in the PD-1 peptide precipitation indicated. (B) In the resting or unstimulated TCR signalosome, Lck is phosphorylated on its negative regulatory tyrosine residue (indicated by the red circle) by PAG/Cbp associated Csk kinase activity. Co-ligation of the TCR with PD-1 results in SHP-2 phosphorylation (and perhaps PD-1) presumably by Lck or Csk, as indicated by dashed lines. PD-1 suppresses TCR signaling likely via a recruited SHP phosphatase activity corresponding with decreased phosphorylation of the CD3ζ ITAM sites, attenuated ZAP70 activation, and inhibition of downstream signal transduction. The phosphorylation sites in the cytoplasmic domains of the indicated receptors and signaling molecules are denoted in black circles or rectangles.

Due to constitutive expression of B7x, the ligand for BTLA, on many tissues types, Zang et al. [30] suggest that BTLA may modulate T-cell responses to tissue specific antigens. CTLA-4 has a role in early T-cell activation, anergy, and self reactive immunity [8,31]. Recently, it has been shown that CTLA-4 deficient T-cells bypass the STAT6 requirement in Th2 differentiation, suggesting that CTLA-4 signaling potentiates the Th2 lineage cytokine signals [32]. PD-1 has a role in dysregulation of self reactive immune cells [4] and positive selection of thymocytes, affecting the mature T-cell repertoire [5]. BTLA, CTLA-4, and PD-1 are likely contributing to distinct immunomodulatory roles during development, antigen challenge, and disease. For each of these molecules, the receptor proximal recruitment of tyrosine phosphatases appears to be a key signaling mechanism. We show here that PD-1 inhibition of T-cells also involves SHP1/2 association. More significantly, PD-1 mediated inhibitory signal blocks the ZAP70/CD3ζ signalosome resulting in attenuated PKCθ activation and signaling to IL-2 production.

Acknowledgements: We very gratefully acknowledge anti-PD-1 reagent gift from Dr. T. Honjo (Kyoto University). The MSCV2.2-derived retroviral vector was very kindly provided by Dr. Kenneth Murphy (Washington University).

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