Fyn and PTP-PEST–mediated Regulation of Wiskott-Aldrich Syndrome Protein (WASp) Tyrosine Phosphorylation Is Required for Coupling T Cell Antigen Receptor Engagement to WASp Effector Function and T Cell Activation

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Abstract

Involvement of the Wiskott-Aldrich syndrome protein (WASp) in promoting cell activation requires its release from autoinhibitory structural constraints and has been attributed to WASp association with activated cdc42. Here, however, we show that T cell development and T cell receptor (TCR)-induced proliferation and actin polymerization proceed normally in WASp−/− mice expressing a WASp transgene lacking the cdc42 binding domain. By contrast, mutation of tyrosine residue Y291, identified here as the major site of TCR-induced WASp tyrosine phosphorylation, abrogated induction of WASp tyrosine phosphorylation and its effector activities, including nuclear factor of activated T cell transcriptional activity, actin polymerization, and immunological synapse formation. TCR-induced WASp tyrosine phosphorylation was also disrupted in T cells lacking Fyn, a kinase shown here to bind, colocalize with, and phosphorylate WASp. By contrast, WASp was tyrosine dephosphorylated by protein tyrosine phosphatase (PTP)-PEST, a tyrosine phosphatase shown here to interact with WASp via proline, serine, threonine phosphatase interacting protein (PSTPIP)1 binding. Although Fyn enhanced WASp-mediated Arp2/3 activation and was required for synapse formation, PTP-PEST combined with PSTPIP1 inhibited WASp-driven actin polymerization and synapse formation. These observations identify key roles for Fyn and PTP-PEST in regulating WASp and imply that inducible WASp tyrosine phosphorylation can occur independently of cdc42 binding, but unlike the cdc42 interaction, is absolutely required for WASp contributions to T cell activation.

Key words: WASp • lymphocyte activation • tyrosine phosphorylation • actin cytoskeletal arrangement

Introduction

The Wiskott-Aldrich syndrome protein (WASp) is a hematopoietic lineage-restricted cytosolic effector of T cell activation, integrally involved in the coupling of TCR engagement to transcriptional activation and cytoskeletal rearrangement (1). WASp effects on TCR signaling are mediated through its tandemly located GTPase binding domain (GBD) and proline-rich region, flanking NH2-terminal–located Ena/Vasp homology 1, and COOH-terminal verprolin homology central region–acidic region (VCA) domains. The VCA domain represents a newly identified structural motif that provides WASp and several WASp-related proteins with the capacity to bind components of the Arp2/3 complex and thereby induce Arp2/3 actin-nucleating activity (2,3). Affinity of this domain for Arp2/3 complex binding has very recently been shown to

Abbreviations used in this paper: GBD, GTPase binding domain; GFP, green fluorescent protein; GST, glutathione S-transferase; PKC, protein kinase C; PSTPIP, proline, serine, threonine phosphatase interacting protein; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; pTyr, phosphotyrosine; VCA, verprolin homology central region–acidic region; WASp, Wiskott-Aldrich syndrome protein.
be enhanced by its constitutive serine phosphorylation (4). The other functional domains of WASp complement this activity by mediating effector interactions that enable WASp recruitment into TCR signaling pathways and the consequent linkage of TCR stimulatory signals to induction of actin polymerization. For example, TCR-stimulated binding of the WASp proline-rich region to the SH3 domain–containing proline, serine, threonine phosphatase interacting protein (PSTPIP)1 adaptor, allows WASp to be targeted to the T cell–APC interface and thereby direct the regional actin cytoskeletal rearrangements required for formation of the immunological synapse (5). Similarly, WASp proline-rich region–mediated interactions with other SH3 domain–containing adaptors, such as CrkL and intersectin 2, and WASp Ena/Vasp homology 1 domain–mediated interactions with the WASp–interacting protein, WIP, appear to enable WASp translocation to specific subcellular sites wherein its actin polymerizing properties can be deployed so as to augment cell activation (6–9). Such interactions may also account for the capacity of WASp to mediate lipid raft clustering after TCR engagement (10). By contrast, the WASp GBD mediates an interaction with the activated form of the cdc42 Rho GTPase that does not modulate WASp localization, but instead induces WASp actin polymerizing function by releasing WASp from an autoinhibitory structural configuration that constrains VCA effects on the Arp2/3 complex (11).

The structural and biochemical data showing WASp actin polymerizing properties to be constitutively, but reversibly, autoinhibited, provide a mechanism explanation for the inducibility of actin cytoskeletal change in response to cell stimulation (12, 13). However, data showing that WASp promotes clustering in the absence of cdc42 binding imply that WASp Arp2/3 stimulatory function can be evoked by mechanisms other than cdc42–GTP association with its GBD (13). This possibility is also suggested by nuclear magnetic resonance data showing that a WASp tyrosine residue (Y291) in close proximity to the WASp GBD is structurally positioned such that its phosphorylation might disrupt WASp autoinhibitory conformation and thereby evoke VCA domain activity (12). This tyrosine residue has been shown to be phosphorylated in vitro by the protein tyrosine kinases (PTKs) Btk and Hck (14, 15), and its phosphorylation has been associated with enhanced macrophage filopodium formation (15). However, although WASp has also been shown to interact with Fyn and Itk (16, 17), the regulation of its tyrosine phosphorylation and the relevance of WASp tyrosine phosphorylation to its biological functions in T cells remain unknown.

In this study, the sites, mechanisms, and biologic relevance of WASp tyrosine phosphorylation in relation to T cell activation were investigated. The data indicate inducible phosphorylation of the WASp tyrosine residue at position 291 to be essential for the coupling of TCR engagement to NFAT activation, actin polymerization, and immunological synapse formation. By contrast, WASp effects on T cell development and activation as well as its inducible tyrosine phosphorylation can occur independently of the WASp GBD motif. The data also identify Fyn as the WASp–binding PTK required for phosphorylation of Y291 after TCR stimulation and indicate that WASp association with the PSTPIP1 adaptor allows WASp to colocalize with and be dephosphorylated by the protein tyrosine phosphatase (PTP), PTP-PEST. Like WASp, both Fyn and PTP-PEST are inducibly translocated to the contact zone formed between T cells and stimulatory APCs, but although WASp and Fyn are required for immunological synapse formation, PTP-PEST overexpression essentially abrogates synapse development. Similarly, Fyn markedly enhances, whereas PTP-PEST in combination with PSTPIP1 impairs, WASp capacity to stimulate Arp2/3 actin polymerizing activity in vitro. Importantly, Fyn effects on WASp phosphorylation and its actin polymerizing role appear to be unaffected by changes in cdc42 activity and disruption of the WASp–cdc42 interaction, respectively. These findings indicate Y291 phosphorylation to be essential to the triggering of WASp effector function during T cell activation. The data also reveal that WASp tyrosine phosphorylation can occur independently of WASp–cdc42 interaction and its modulation represents a key mechanism whereby Fyn and PTP-PEST regulate the signaling events linking TCR ligation to transcriptional activation and cytoskeletal rearrangement.

Materials and Methods

Mice. To generate WASpΔGBD mice, a WASp cDNA deleted for sequences encoding amino acids 238–252 (WASpΔGBD) was introduced downstream of the CD2 promoter elements in the p29Δ2 vector and the transgene construct was then injected into CD1 embryos. Transgene’ founders were identified by Southern analysis and PCR using primer pairs: 5’–GGAGCA- CATCAG AAGGGCTGGCTT-3’ (forward) and 5’CGGAGG-AACCAGAGTGGACCT-3’ (reverse). Founderes were then backcrossed to the C57BL/6 background and pups were screened for WASpΔGBD expression by immunoblotting analysis of lymphoid cell lysates using anti-WASp antibody. Selected WASpΔGBD transgenic lines were then mated with WASp-deficient (WAS−/−) mice (18) to derive animals expressing WASpΔGBD on the WAS−/− background (WAS−/−ΔGBD). Mice expressing the OVA-specific OT-II TCR transgene on the C57BL/6 background (provided by F. Carbone, University of Melbourne, Melbourne, Australia, and T. Watts, University of Toronto, Toronto, Canada; reference 19) were bred with WASpΔGBD/ΔGBD mice to generate WAS−/−/OT-II, WAS−/−ΔGBD/OT-II, and Fyn−/−/OT-II animals. C57BL/6 Itk−/− mice were provided by D. Fowell (University of Rochester Medical Center, Rochester, NY).

Reagents. Reagents used for these studies included: polyclonal antibodies specific for WASp (raised against a synthetic peptide representing WASp residues 224–238), PSTPIP1 (5), and PTP-PEST (provided by M. Tremblay, McGill University, Montreal, Canada, and A. Veillette, Clinical Research Institute of Montreal, Montreal, Canada); monoclonal antibodies recognizing human and murine CD3 (from the OKT3 and 2C11 hybridomas, respectively), human and murine CD28 (from the 9.2 and 37.51 hybridomas, respectively), and human protein kinase C (PKC)-θ.
blocking with 5% skim milk powder in 1X Tris buffered saline-Tween (TBS-T) (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.25 mM Na3VO4, 0.5% Nonidet P-40, 0.1 mM 2-mercaptoethanol) and the complexes were incubated for 30 min at 30°C in kinase buffer containing 10 μg/mL [γ-32P]ATP (NEN Life Science Products) with 5 μg GST, GST-WASp, GST-WASpΔGBD, or GST-WASpΔPro fusion proteins. Samples were resuspended in Laemmli buffer and resolved in 10% SDS-PAGE gels. After electrophoresis and transfer to nitrocellulose, phosphorylated WASp was visualized by autoradiography.

NFAT Luciferase Reporter Assay. Murine WAS<sup>−/−</sup> thymocytes (2 × 10<sup>6</sup> cells) were isolated and transfected with 50 μg of an NFAT luciferase reporter gene plus 50 μg pEFGP-C3 vectors encoding WASp-GFP, WASp-Y<sup>→</sup>F GFP mutants, or GFP alone. After 4 h, viable cells were separated using Lympholyte M (Cedarlane) and the GFP<sup>+</sup> cells were purified over a MoFlo<sup>®</sup> cell sorter (DakoCytomation) and cultured for 8 h in uncoated 96-well plates or in plates coated with 10 μg/ml anti-CD3 or 5 μg/ml anti-CD28 plus anti-CD28 antibody. Cells were lysed and luciferase activity was assayed using the luciferase assay system (Promega) and a luminometer.

Synapse Assay. LB27.4 B cells (H–2<sup>b</sup>-restricted B cell hybrid, American Type Culture Collection) were incubated with OVA peptide (OVA<sub>229-239</sub>) for 4 h at 37°C followed by centrifugation at 200 g for 5 min with an equal number of lymph node T cells from transgenic mice or from WAS<sup>−/−</sup>/OT-II lymphocytes transfected with pEFGP-C3 or pDSRED expression constructs. Samples were incubated at room temperature for 10 min and the cells were resuspended and plated onto poly-L-lysine–coated coverslips (Biocoat; Becton Dickinson) before fixation in 3% paraformaldehyde. Synapse formation was scored as the percent conjugates (T cell in physical contact with an APC) showing actin accumulation at the T cell–APC interface.

Transfection and Immunofluorescence Assays. Plasmid DNA for expression constructs containing PFP1P1, PTP-PEST, WASp,
WASpY291F, and WASpY102F cDNAs were purified using CLONTECH Laboratories, Inc. Maxi-Prep kit. 5 × 10^5 Cos-7 cells maintained in DMEM supplemented with 10% fetal bovine serum, 1-glutamine, and penicillin/streptomycin, were seeded onto glass coverslips and transfected with selected plasmid DNA using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, cells were washed and fixed with 3% ice-cold paraformaldehyde in PBS. Alternatively, unstimulated or stimulated OT-II T cells were transfected by electroporation (1 pulse, 360 mV) using a BTX electroporator and subjected to fixation at 2.5 h after transfection. After fixation, cells were blocked with 2% BSA/PBS for 10 min immediately or for intracellular staining, cells were first permeabilized with 0.1% Triton X-100/PBS. Cells were then incubated with primary and the appropriate fluorescently conjugated secondary antibodies and the stained samples were mounted in anti-fade mounting media (DakoCytomation). Images were analyzed using the Olympus IX-70 inverted microscope equipped with fluorescence optics and Deltavision Deconvolution Software (Applied Precision).

**In Vitro Actin Polymerization Assay.** Actin polymerization was evaluated by assaying increase in fluorescence of pyrene-labeled actin using the actin polymerization kit from Cytoskeleton, Inc. for 10 min immediately or for intracellular staining, cells were first transfected by electroporation (1 pulse, 360 mV) using a BTX electroporator and subjected to fixation at 2.5 h after transfection. After fixation, cells were blocked with 2% BSA/PBS for 10 min immediately or for intracellular staining, cells were first permeabilized with 0.1% Triton X-100/PBS. Cells were then incubated with primary and the appropriate fluorescently conjugated secondary antibodies and the stained samples were mounted in anti-fade mounting media (DakoCytomation). Images were analyzed using the Olympus IX-70 inverted microscope equipped with fluorescence optics and Deltavision Deconvolution Software (Applied Precision).

**In Vivo Actin Polymerization Assays.** 1.5 × 10^6 thymocytes obtained from WAS^−/−ΔGBD mice or by transfection of WAS^−/− thymic cells with the pEGFP-C3 WASp Y→F mutant constructs were either unstimulated or stimulated with anti-CD3 and anti-CD28 antibodies for 30 min on ice followed by cross-linking with 5 μg mouse anti–hamster IgG for 5 min at 37°C. Activation was terminated and the cells were fixed in 5% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 5 min on ice, washed and stained with 5 μg/ml FITC-conjugated phalloidin, and analyzed using a FACSCalibur™.

**Figure 1.** The GBD of WASp is not essential for WASp activity. (A) Scheme that shows the domain organization of wild-type and a GBD-deleted (WASpΔGBD) WASp cDNA used to derive WAS^−/−ΔGBD mice. Positions of all tyrosine residues within WASp are indicated. (B) Lysates obtained from WASp or WASpΔGBD-expressing Cos-7 cells were incubated with GTPγS-loaded GST-cdc42 fusion protein bound to glutathione sepharose beads and the complexes were then resolved by SDS–PAGE and sequential immunoblotting with anti-WASp and anti-cdc42 antibodies (top two panels). Immunoblotting analysis demonstrating WASp and WASpΔGBD expression in Cos-7 transfectants (Tfn) is shown in the bottom panel. (C) Immunoblotting showing the WASp species detected in thymocytes and lymphocytes from wild-type (WT), WAS^−/−, and WAS^−/−ΔGBD mice using an anti-WASp antibody. (D) Lymphocytes and thymocytes isolated from 4–8-wk-old wild-type (WT), WAS^−/−, and WAS^−/−ΔGBD mice were cultured for 48 h in medium alone or with either 1 μg/ml anti-CD3 antibody or 0.2 μg/ml anti-CD3 plus anti-CD28 antibody. Antigen receptor-evoked proliferative responses were determined after an 18-h pulse with [3H]thymidine. Values represent the means (± SEM) of four independent experiments. (E) Thymocytes from wild-type (WT), WAS^−/−, and WAS^−/−ΔGBD mice were isolated and stimulated with anti-CD3 plus anti-CD28 antibodies for 30 min on ice followed by cross-linking with a secondary antibody. Cells were fixed with 5% paraformaldehyde and F-actin content was quantified by flow cytometric analysis of FITC phalloidin–stained resting (bold lines) and stimulated (dashed lines) cells. The results are representative of three independent experiments.
Results and Discussion

TCR-induced Proliferation and Actin Polymerization Proceed Normally in WAS−/−ΔGBD Mice. Although activated cdc42 can bind WASp and trigger WASp VCA domain–mediated Arp2/3 activity (2, 3), it is unclear whether this interaction is required and/or solely responsible for induction of WASp effector function after TCR stimulation. To address this issue, the relevance of the GBD–cdc42 interaction to WASp effects on T cell activation was studied using mice in which a CD2 promoter/enhancer-driven WASp cDNA lacking the GBD (WASpΔGBD; Fig. 1A) was expressed on the C57BL/6 WAS−/− background. After confirmation that the WASpΔGBD species does not bind activated cdc42 (Fig. 1B) and demonstration that the WASpΔGBD transgene is expressed in T lineage cells (Fig. 1C) and that these mice reveal CD3/...
CD28-induced actin polymerization, which is abrogated in WAS\(^-/-\) cells, to be essentially normal in WAS\(^-/-\)ΔGBD cells (Fig. 1 E). Together, these observations indicate the coupling of TCR engagement to proliferation and actin polymerization to be unaffected by absence of the WASp GBD and thus imply that WASp effects on TCR-induced transcriptional activation and cytoskeletal arrangement can be evoked by mechanisms other than cdc42–GBD interaction.

Phosphorylation of WASp Tyrosine 291 Is Induced by TCR Engagement and Required for Induction of NFAT Translocation, Actin Polymerization, and Synapse Formation. The negligible overt T cell functional deficit observed in WAS\(^-/-\)ΔGBD mice implies that WASp activation in T cells might be mediated through non-GBD–dependent mechanisms. Data showing WASp or its orthologue, N-WASP, to undergo inducible tyrosine phosphorylation in B cells, platelets, and neurons (14, 21, 22) raise the possibility that TCR–evoked WASp tyrosine phosphorylation is relevant to the regulation of WASp function in T cells. To determine whether WASp is inducibly tyrosine phosphorylated after T cell stimulation, WASp immunoprecipitates from resting and TCR–stimulated Jurkat cells were subjected to anti-pTyr immunoblotting analysis. This analysis revealed WASp to be tyrosine phosphorylated constitutively in these cells, but to undergo a marked increase in tyrosine phosphorylation after TCR engagement (Fig. 2 A). Moreover, both basal and TCR–evoked WASp tyrosine phosphorylation levels appeared equivalent in thymocytes from wild-type and WAS\(^-/-\)ΔGBD mice, suggesting that in T cells, WASp can be inducibly tyrosine phosphorylated in the absence of its interaction with activated cdc42 (Fig. 2 B). To determine which of the seven tyrosine residues within WASp (Fig. 1 A) undergo TCR-induced phosphorylation in vivo, cDNAs in which each tyrosine residue was individually replaced with phenylalanine (Y→F) were expressed as GFP-tagged proteins in Jurkat cells and phosphorylation status of the mutant proteins was examined after cell stimulation. As revealed by anti-pTyr immunoblotting analysis of anti-GFP immunoprecipitates from the transfected cells, induction of WASp tyrosine phosphorylation was unaffected by mutation at six of the seven WASp tyrosine sites, but was abrogated in cells expressing WASpY291F (Fig. 2 C). These findings indicate that TCR engagement triggers the tyrosine phosphorylation of WASp and demonstrate that Y291, a residue known to be selectively targeted by Btk (14), represents the major and likely only tyrosine site on WASp targeted for phosphorylation after TCR stimulation.

To examine the biologic relevance of WASp tyrosine phosphorylation in T cells and specifically determine whether Y291 phosphorylation modulates WASp function, WAS\(^-/-\) T cells were reconstituted with the WASp Y→F mutants and the cells were then assayed for NFAT transcriptional activity and actin polymerization. As revealed using an NFAT luciferase reporter, induction of NFAT activity after CD3 or CD3/CD28 stimulation was profoundly reduced in WASp Y291F relative to wild-type WASp–expressing cells, but unaffected in the context of other WASp tyrosine mutations (Fig. 2 D). Similarly, immunofluorescence analysis of phalloidin-labeled WAS\(^-/-\) thymocytes expressing either wild-type or tyrosine-substituted WASp mutants, revealed induction of actin polymerization to be disrupted by either WASp deficiency or WASpY291F expression (Fig. 2 E), but to proceed normally in cells expressing wild-type WASp, WASpY88F, WASpY212F (Fig. 2 E), or any of the other WASp mutants (not depicted). Therefore, Y291 phosphorylation modulates and is, in fact, required for WASp contribution to the induction of transcriptional activation and actin polymerization after TCR engagement.

The critical role for WASp Y291 phosphorylation in linking TCR engagement to actin polymerization implies that WASp effects on actin-mediated processes associated with T cell activation are also dependent on its phosphorylation at this site. To address this possibility, the effects of Y291F mutation on WASp capacity to promote immunological synapse formation after TCR stimulation were investigated using T cells from WAS\(^-/-\) mice expressing a TCR transgene (OT-II) that specifically recognizes an epitope of OVA. For those studies, WAS\(^-/-\)/OT-II T cells transfected with wild-type or the tyrosine-mutated WASp cDNAs were stimulated with OVA-pulsed APCs and synapse formation was then evaluated using immunofluorescence microscopy to monitor accumulation of polymerized actin and PKC-θ at the T cell–APC interface of developing conjugates. As is consistent with the essential role identified for WASp in synapse development (5), actin and PKC-θ accumulation indicative of synapse formation at the T cell–APC contact site was absent in WAS\(^-/-\)/OT-II cells, but was fully restored by expression of wild-type WASp (Fig. 2 F, a and b). Induction of synapse formation in these cells was also restored by expression of WASp Y102F (Fig. 2 F, c) and all other WASp tyrosine mutants (not depicted) with the exception of WASpY291F, the latter of which translocated to the synaptic region after cell stimulation, but failed to evoke the actin reorganization and PKC-θ localization within this region that demarcate synapse formation (Fig. 2 F, d). These data suggest that WASp translocation to the T cell–APC contact zone occurs independently of its tyrosine phosphorylation, whereas formation of the immunological synapse after TCR engagement requires not only WASp, but also phosphorylation of WASp at Y291. Together, these findings reveal phosphorylation at the Y291 site to be critical for activation of WASp effector function in T cells and thus imply that this modification enables the release of WASp from autoinhibitory structural constraints.

Tyrosine Phosphorylation of WASp Is Regulated by Fyn and PTP-PEST. Phosphorylation of WASp at Y291 by both Btk and Hck has been demonstrated in exogenous expression systems (14, 15), but the PTKs modulating Y291 phosphorylation in vivo in T cells have not been defined. Similarly, although WASp has been shown to bind GST fusion proteins containing the Itk, Lck, or Fyn SH3 domains and associate with Fyn in transformed monocytes
the relevance of these interactions to the in vivo induction of WASp tyrosine phosphorylation in T cells remains unknown. To address these issues, induction of WASp tyrosine phosphorylation was evaluated in T cells deficient for Itk, Lck, or Fyn. As shown in Fig. 3 A, immunoblotting analysis revealed no differences in the levels of WASp tyrosine phosphorylation elicited in T cells from Itk-deficient compared with wild-type mice. Similarly, comparisons between Lck-deficient JCam-1 cells and JCam-1 cells reconstituted for Lck expression revealed induction of WASp tyrosine phosphorylation to be unaffected by Lck deficiency. By contrast, T cells from Fyn-deficient mice showed no detectable increase in tyrosine phosphorylation in response to TCR engagement. Moreover, TCR-elicited WASp tyrosine phosphorylation was markedly lower in Jurkat cells coexpressing wild-type and a catalytically inert Fyn (FynK296M) protein (23) compared with cells expressing only wild-type Fyn and was essentially abrogated by the sole overexpression of FynK296M (Fig. 3 B). These data indicate that Fyn, but not Itk or Lck, is required for WASp tyrosine phosphorylation and raise the possibility that WASp might be exclusively tyrosine phosphorylated by Fyn in T cells. Importantly, the coexpression in Jurkat cells of Fyn with either dominant negative (cdc42-N17) or activated (cdc42-V12) cdc42 mutant proteins had no effect on TCR-induced WASp tyrosine phosphorylation, implying that Fyn phosphorylates WASp independently of cdc42 activity (Fig. 3 C).

Because these findings suggest that WASp is a Fyn substrate, Fyn association with WASp was evaluated in Jurkat cells. As shown in Fig. 3 D, these proteins inducibly interact with one another after CD3/CD28 stimulation. The
capacity of Fyn to bind directly to WASp was also examined by incubating polyhistidine-tagged Fyn (His Fyn) with glutathione sepharose–bound GST fusion proteins containing wild-type WASp or a WASp mutant lacking the proline-rich region (WASpΔPro). Anti-Fyn immunoblotting analysis of these complexes revealed interaction of His Fyn proteins with the GST–WASp fusion protein, but not with control GST or GST–WASpΔPro proteins (Fig. 3E). Thus, the association of Fyn with WASp is direct and requires the WASp proline-rich region and its likely binding to the Fyn SH3 domain.

To determine whether Fyn directly phosphorylates WASp, the effects of Fyn on the WASp phosphorylation state were assessed using an in vitro kinase assay. As shown in Fig. 3F, Fyn immunoprecipitates from activated T cells induced the tyrosine phosphorylation of WASp, but neither WASpΔPro nor WASpY291F mutant proteins were phosphorylated in this assay. These findings suggest that

Figure 4. WASp inducibly associates and colocalizes with PSTPIP1 and PTP-PEST. (A) Jurkat T cells were stimulated for the indicated times with anti-CD3 and anti-CD28 antibodies and lysates were then prepared and immunoprecipitated with anti-PSTPIP1 antibody. The immune complexes were subjected to SDS-PAGE and sequentially immunoblotted with anti-PST-PEST, anti-WASp, and anti-PSTPIP1 antibodies. (B) Jurkat T cells were stimulated with anti-CD3 and anti-CD28 antibodies and lysates were then prepared and immunoprecipitated with anti-WASp antibody. Complexes were resolved by SDS-PAGE followed by sequential immunoblotting with anti-PTP-PEST and anti-WASp antibodies. (C) Lysates prepared from Jurkat T cells were incubated with GST, GST-PSTPIP1, full-length (FL), GST–PSTPIP1COIL, or GST–PSTPIP3SH3 fusion proteins bound to glutathione–sepharose beads. Complexes were resolved by SDS-PAGE and immunoblotted using an anti–PTP-PEST antibody. (D) Cos-7 cells were transiently transfected with pEGFP-PSTPIP1 (a), pEGFP-PTP-PEST (b), pEGFP-WASp (c), pEGFP-PSTPIP1 and pcDNA3-PTP-PEST (d), or pEGFP-PSTPIP1, DSRRED–WASp, and pcDNA3-PTP-PEST (e). Cells were fixed, stained with rhodamine phalloidin for actin (a–c) or with anti–PTP-PEST antibody (d and e) and Cy5 anti–rabbit Ig (e), and then analyzed by confocal immunofluorescent microscopy. The images shown are representative of three independent experiments. (E) pcDNA3 constructs for expression of wild-type or catalytically inactive (C231S) PTP-PEST were cotransfected with pEGFP-WASp (WASp-GFP) into Jurkat cells. The cells were either left unstimulated or stimulated with anti-CD3 and anti-CD28 antibodies, lysed, and the lysate proteins were immunoprecipitated with anti-GFP antibodies. The complexes were subjected to SDS-PAGE and immunoblotted sequentially with anti–p-Tyr and anti-GFP antibodies.

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Fyn-mediated WASp phosphorylation requires the physical association of these proteins and that it is Y291 and no other WASp tyrosine residue that is targeted by Fyn during T cell activation.

The relationship between tyrosine phosphorylation and WASp effector activity in T cells implies that PTP-mediated dephosphorylation of WASp is also important to WASp functions in T cell activation. Previous studies of WASp ligands in T cells have revealed that WASp inducibly associates with a cytosolic adaptor, PSTPIP1, also known to bind a hemopoietic PTP, PTP-PEST (5, 24). These observations raise the possibility that WASp might be juxtaposed to and thereby dephosphorylated by PST-PEST via its association with PSTPIP1, a paradigm observed in relation to PTP-PEST-mediated dephosphorylation of the c-Ab1 PTK (25).

To determine whether these effectors associate with one another in T cells, PSTPIP1 immunoprecipitates from CD3/CD28-stimulated T cells were examined for the presence of WASp and PTP-PEST. As shown in Fig. 4 A, both WASp and PTP-PEST coimmunoprecipitated with PSTPIP1. PTP-PEST was also detected in WASp immunoprecipitates from stimulated T cells (Fig. 4 B), but recombinant WASp and PST-PEST did not associate in an in vitro binding assay, suggesting that their interaction is indirect and possibly mediated via PSTPIP1. To address this possibility and extend data on the structural basis for PSTPIP1–PTP-PEST interaction (26), GST fusion proteins containing full-length PSTPIP1 or either of its major protein-binding domains (a coiled coil and an SH3 domain) were assessed for capacity to precipitate PTP-PEST from stimulated T cells. Results of this analysis revealed the interaction of PTP-PEST with GST fusion proteins containing full-length PSTPIP1 or the PSTPIP1 coiled coil region alone, but not with GST-PSTPIP1 SH3 domain fusion protein (Fig. 4 C). As PSTPIP1 has previously been shown to bind via its SH3 domain to WASp (5), these findings suggest that WASp and PST-PEST interact via their mutual binding to PSTPIP1. Because these three proteins have each been implicated in actin cytoskeletal rearrangement (1, 23, 27), the functional relevance of a trimolecular WASp–PSTPIP1–PTP-PEST interaction was investigated using fluorescence microscopy to evaluate the localization of these proteins with respect to one another and to actin. As shown in Fig. 4 D, rhodamine phalloidin staining of Cos-7 cells expressing GFP-tagged WASp, PSTPIP1, or PTP-PEST revealed each of these proteins to be highly or entirely colocalized with actin structures, WASp being concentrated in actin-rich perinuclear aggregates, and PSTPIP1 and PTP-PEST localizing to actin filibrillary networks spanning the cytoplasm. Coexpression of PSTPIP1 and PTP-PEST revealed these proteins to be colocalized and distributed similarly as when expressed alone (Fig. 4 D, d). When coexpressed with PSTPIP1 and PTP-PEST, WASp also showed a distribution pattern overlapping that of PSTPIP1/PTP-PEST (Fig. 4 D, e). Thus, as shown for PSTPIP1 (5), PSTPIP1/PTP-PEST association with WASp appears to evoke WASp relocalization such that these three effectors colocalize within the actin cytoskeleton, as is suggestive of a biological role for PSTPIP1-mediated linkage of WASp to PTP-PEST.

In view of its capacity to associate and colocalize with PTP-PEST, the potential for WASp to serve as a PTP-PEST substrate in T cells was examined by coexpressing WASp with PTP-PEST or a catalytically inactive form of PTP-PEST (PTP-PEST C231S) in Jurkat cells and examining these cells with respect to inducible WASp tyrosine phosphorylation status. As shown in Fig. 4 E, TCR-induced WASp tyrosine phosphorylation in these cells is essentially abrogated by PTP-PEST overexpression, but is enhanced by expression of catalytically inactive and likely dominant negative–acting PTP-PEST C231S. Thus, PTP-PEST-mediated tyrosine dephosphorylation may counteract Fyn-mediated phosphorylation of WASp after T cell stimulation and, by extension, WASp contributions to T cell activation might be modulated by the relative balance of these respective enzyme activities.

Fyn and PTP-PEST Modulate WASp Effects on Induction of Actin Polymerization and Immunological Synapse Formation. In view of the effects of Fyn and PTP-PEST on WASp tyrosine phosphorylation and relevance of Y291 phosphorylation to WASp function, the possibility that Fyn and/or PTP-PEST modulate the ability of WASp to activate the Arp2/3 complex was directly tested using a pyrene fluorescence in vitro assay of actin polymerization. As shown in Fig. 5 A, WASp alone stimulated some polymerization of the pyrene-labeled actin, but the effect of WASp on actin polymerization was dramatically increased by the addition of Fyn. Because WASp effector activity can also be stimulated by cdc42 (1, 3), the efficacies of Fyn and cdc42 in triggering either WASp or WASpΔGBD-mediated actin polymerization were also compared using a GST fusion protein containing constitutively active cdc42 (cdc42-V12). Although GST–cdc42-V12 substantively enhanced WASp-mediated actin polymerization, when added at a concentration generating maximum Arp2/3 activation, its effects on actin polymerization were no greater than those induced by Fyn and were not increased by the addition of GST–Fyn (Fig. 5 A). Similarly, the addition of either GST–Fyn or GST–cdc42-V12 alone and in combination at concentrations yielding lower levels of WASp–Arp2/3 activation revealed the effects of cdc42-V12 and Fyn together to be less than additive (Fig. 5 B). Thus, although Fyn and cdc42 both stimulate WASp-mediated actin polymerization, they do not act synergistically to activate WASp. By contrast, the addition of PTP-PEST and PSTPIP1 had a dramatic effect on Fyn’s capacity to stimulate WASp activity with the level of WASp–Arp2/3 activity evoked by the combination of Fyn with PTP-PEST and PSTPIP1 being comparable to that triggered by WASp alone and being even further diminished by the combination of PTP-PEST and PSTPIP1 (Fig. 5 A). Thus, as is consistent with a key role for tyrosine phosphorylation in regulating WASp function, these data reveal WASp’s effects on actin polymerization to be modulated by both Fyn and PTP-PEST/PSTPIP1. The data also reveal Fyn to be as efficacious as
Figure 5. Fyn and PTP-PEST modulate WASp effects on induction of actin polymerization and synapse formation. (A) Polymerization of 2.8 μM pyrene-labeled actin monomer was assayed in the presence of 20 nM Arp2/3 complex, 100 nM GST–WASp or GST–WASpΔGBD, and GST fusion proteins containing 50–500 nM either Fyn, PTP-PEST, PSTPIP, or cdc42-V12. Polymerization was monitored by the increase in prenyl-actin fluorescence. B. The pyrene actin assay was used to compare the WASp-Arp2/3–actin polymerizing activities of cdc42 at low (15 nM), medium (250 nM), or high (500 nM) concentration and Fyn at low (10 nM), medium (100 nM), or high (200 nM) concentration alone or in combination. (C) Fyn effects on synapse formation were evaluated by incubating lymphocytes from OT-II (a and b) and Fyn⁻/⁻/OT-II (c) mice with unpulsed (a) or OVA peptide–pulsed (b and c) LB27.4 cells followed by cell fixation, staining for WASp, Fyn, and actin, and visualization by immunofluorescent microscopy. Images on the far left of each panel represent a merge of the other three images within each panel. A computer-generated three-dimensional reconstruction of the synaptic region formed between wild-type T cells and APCs (d) shows the localization of Fyn in the central area of the synapse and the distribution of WASp in both the central and peripheral synaptic region. Synapses were quantified (e) by counting the numbers of T cell–APC conjugates showing clustered actin at the conjugation site. Values shown are the percent of conjugates with synapse formation and represent means (± SEM) of three independent experiments. (D) PTP-PEST effects on synapse formation were assessed using WASp⁻/⁻/OT-II lymphocytes transfected with pDSRED-WASp (a), pcDNA3–WASp and pDSRED-PSTPIP1 (b), or pcDNA3–WASp, pEGFP-PSTPIP1, and pDSRED-PTP-PEST (c). Cells were incubated with OVA peptide–pulsed LB27.4 B cells, fixed, and stained for actin and/or PKC-θ, and visualized by immunofluorescent microscopy. The image on the far right of each panel is a merged image of all other images in the panel. Synapses were quantified by counting the number of T cell–B cell conjugates showing clustered actin at the synaptic site. Values shown are the percent of conjugates with synapse formation and represent the means (± SEM) of three independent experiments.
cdc42 in stimulating WASp-Arp2/3 activity and modulating this activity independently of the WASp GBD.

To directly establish the relevance of Fyn and PTP-PEST to WASp functions in T cells, the capacity of these effectors to modulate WASp effects on immunological synapse formation was also investigated using the OT-II transgenic mice as well as mice expressing the OT-II transgene on the Fyn \(^{-/-}\) background. As shown in Fig. 5 C, stimulation of T cells from OT-II TCR mice with OVA peptide–pulsed APCs induced translocation of both WASp and Fyn to the T cell–APC interface where they colocalized with a region of intense actin accumulation demarcating the developing synapse. Three-dimensional reconstruction and 90\(^\circ\) rotation of the interface area confirmed published data indicating Fyn to be concentrated centrally within the synapse (28) and revealed WASp to be distributed between both the central and peripheral synaptic region. By contrast, synapse formation, as revealed by actin accumulation at the T cell–APC interface, was essentially not detectable in conjugates formed between Fyn \(^{-/-}\)/OT-II cells and antigen–pulsed APCs (Fig. 5 C, c and e). Synapse formation was also found to be impaired by the coexpression of PTP-PEST with WASp in WAS \(^{-/-}\)/OT-II cells with percentages of synapses formed being at least 50\% lower than observed between APCs and WASp-expressing cells (Fig. 5 D, d). As is consistent with the role of PSTPIP1 in coupling PTP-PEST to WASp, the coincident expression of WASp, PSTPIP1, and PTP-PEST in these cells had an even more deleterious effect on synapse formation and, as with Fyn deficiency, abrogated actin accumulation at the T cell–APC interface (Fig. 5 D, c and d). These results are in agreement with published data indicating that Fyn and PSTPIP1 translocate to the T cell–APC contact region after TCR stimulation (5, 28) and suggest that Fyn and PSTPIP/PTP-PEST colocalization with WASp within this region influences WASp capacity to evoke the actin polymerization and cytoskeletal change required for synapse formation. Thus, these data provide further evidence that Fyn and PTP-PEST effects on the tyrosine phosphorylation of WASp are critical to the induction of WASp effector activities required for T cell activation.

The data reported here identify phosphorylation at Y291 as a major mechanism for induction of WASp effector activity after TCR engagement. A requirement for WASp “activation” in T cells is consistent with cumulative data revealing WASp to be constitutively autoinhibited (1–3) and identifying induction of actin polymerization as a critical facet of the T cell response to antigen stimulation (29). The current data identifying Fyn as the PTK responsible for WASp phosphorylation in T cells and implicating PTP-PEST in WASp dephosphorylation in these cells are also in agreement with previous data indicating an essential role for Fyn in induction of cytoskeletal reorganization after TCR engagement (30) and demonstrating that PTP-PEST acts as a negative regulator of lymphocyte activation (31). Taken together with the observed capacity of Fyn to promote and PTP-PEST to inhibit WASp stimulatory effects on Arp2/3 polymerizing function, these findings suggest that tyrosine phosphorylation alone can mediate induction of WASp activity after TCR engagement. By contrast, based on the results of in vitro binding assays, it has recently been suggested that tyrosine phosphorylation effects on WASp activity require its binding to cdc42 (32). Although the current data do not preclude cdc42 binding as a mechanism for activating WASp, the normal behavior of the WASpΔGBD T cells studied here together with the catastrophic effects of Y291 mutation on WASp function and T cell activation indicate that the in vivo induction of WASp effector activity absolutely requires WASp tyrosine phosphorylation and can occur independently of cdc42 binding to WASp. These data do not exclude the possibility that the ΔGBD mutation alters WASp structure so as to disrupt normal autoinhibitory constraints on WASp activity. However, no evidence for “constitutive” T cell activation was observed in the WAS \(^{-/-}\) ΔGBD T cells and the data suggest that WASpΔGBD is constitutively inactive and requires an activation signal for function. Further, although Lyn/Btk-mediated phosphorylation of WASp has been reported to require cdc42 (33), the current data revealing Fyn-mediated tyrosine phosphorylation to be unaffected by constitutively active or dominant negative forms of cdc42 and the GBD deletion to have no effect on Fyn-driven WASp-Arp2/3 actin polymerization in vitro, provide compelling evidence to support previous data indicating that cdc42 binding is not essential for triggering WASp activity (13, 15).

The association of Y291 phosphorylation with induction of WASp effector activity raises the possibility that WASp activation also involves its interactions with SH2 domain–containing signaling effectors. Such effectors may include WASp-binding adapters, such as Nck and Grb2, and kinases, such as Fyn, whose interactions with WASp are mediated via SH3 domain binding to the WASp polyproline region, but may also involve SH2 domain binding to the WASp phosphorylated Y291 residue. Phosphorylation at the Y291 site may also enable currently undefined WASp interactions with SH2 domain–containing effectors and thereby broaden the repertoire of WASp binding so as to facilitate WASp activation as well as its participation in TCR–evoked signaling cascades. Similarly, although Fyn appears to represent the major mediator of WASp tyrosine phosphorylation in T cells, the current data do not exclude the possibility that WASp ligands include tyrosine phosphatases, which like PTP-PEST, target WASp for dephosphorylation and functional deactivation. Although these issues require further investigation, the data presented here indicate the modulation of WASp tyrosine phosphorylation by Fyn and PTP-PEST to be critical to the induction of WASp effector activity in T cells and thus identify WASp tyrosine phosphorylation as essential to its contributions to the coupling of TCR engagement to T cell activation.

The authors thank Drs. Deborah Fowell, Michel Tremblay, Andre Veillette, and Tania Watts for kind provision of reagents and Dr. G. Downey for a generous loan of equipment.

This work was supported by grants to K.A. Siminovitch from...
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