Identification of Fyn as the binding partner for the WASP N-terminal domain in T cells

Mitsuru Sato, Ryoko Sawahata, Takato Takenouchi and Hiroshi Kitani
Transgenic Animal Research Center, National Institute of Agrobiological Sciences, 1-2 Ohwashi, Tsukuba, Ibaraki 305-8634, Japan

Correspondence to: H. Kitani; E-mail: kitani@affrc.go.jp
Received 6 August 2010, accepted 24 May 2011

Abstract

Wiskott–Aldrich syndrome protein (WASP) plays important roles in TCR signaling. In transgenic (Tg) mice, over-expression of the WASP N-terminal region (exons 1–5) including the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) homology 1 (EVH1) domain and anti-WASP-EVH1 single-chain variable fragment (scFv) intracellular expressed antibodies (intrabodies) impairs IL-2 production in activated T cells. However, it largely remains unknown that how this domain transduces TCR signaling. Here, we demonstrate for the first time that the WASP N-terminal domain specifically associates with the Fyn SH3 domain; the interaction was uncovered by screening a \( \text{gt11} \) cDNA expression library obtained from the mouse T-cell line KKF. The interaction between Fyn and WASP was inhibited by over-expression of the WASP N-terminal domain and anti-WASP-EVH1 scFv intrabodies in gene-transfected NIH3T3 cells and T cells derived from these Tg mice. WASP-interacting protein binding to the EVH1 domain of WASP was also inhibited in these Tg mice T cells. Furthermore, tyrosine phosphorylation of WASP and nuclear translocation of nuclear factor of activated T cells following TCR stimulation was severely inhibited by over-expression of the WASP N-terminal domain. These observations strongly suggest that the WASP N-terminal domain plays a pivotal role in the TCR signaling cascade by binding to Fyn.

Keywords: Fyn, IL-2 production, TCR signaling, WASP, WIP

Introduction

Wiskott–Aldrich syndrome (WAS) is an X-linked immunodeficiency caused by mutations in the gene encoding the Wiskott–Aldrich syndrome protein (WASP) (1, 2). WASP is predominantly expressed in hematopoietic cells and regulates immune responses, such as the production of IL-2 and reorganization of actin filaments in TCR signaling. T cells from WASP-deficient mice exhibited a marked reduction in antigen receptor capping and actin polymerization induced by TCR stimulation (3, 4). In addition to these cytoskeletal abnormalities, T cells from WAS patients and WASP-deficient mice showed impairment of proliferation and IL-2 production induced by TCR stimulation (3–5).

WASP is composed of several domains, including an N-terminal enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) homology 1 (EVH1) domain [also known as WASP homology 1 (WH1) domain], a GTPase-binding domain, a proline-rich region (PPR) and a C-terminal verprolin/cofilin/acidic domain. The presence of multiple domains suggests that WASP functions to recruit adapter molecules, protein tyrosine kinases and actin-binding proteins, thus connecting tyrosine kinase signaling pathways to cellular motility driven by actin polymerization (6–13).

The majority of missense and nonsense mutations in WAS patients has been mapped to the WASP N-terminal region, including the EVH1 domain, suggesting that this domain is indispensable for WASP functions (14). However, whether dysfunction of the EVH1 domain is relevant to the WAS disease phenotype remains unknown. To investigate further the function of the WASP N-terminal EVH1 domain in the TCR signaling pathway, we previously developed transgenic (Tg) mice that over-expressed WASP exons 1–5 (aa 1-171, designated WASP15). T cells from WASP15 Tg mice were impaired in their proliferative response and IL-2 production following TCR stimulation, due to the dominant negative effects of the over-expressed WASP15. In contrast, antigen receptor capping and actin polymerization were unaffected (15). The functions of the EVH1 domain were further confirmed in Tg mice-expressing single-chain variable fragment (scFv) intracellular expressed antibodies (intrabodies) that specifically bound the EVH1 domain. Anti-WASP-EVH1 scFv inhibited IL-2 production induced by TCR stimulation without affecting cytoskeletal rearrangements in T cells derived from these Tg mice (16). While these results strongly suggested that the WASP N-terminal region was crucial to IL-2...
production, the details of the functions of this domain in the TCR signaling cascade remained to be elucidated.

To clarify the molecular mechanism through the WASP N-terminal domain in TCR signaling, we sought to isolate molecules that associate with this domain by screening of the λgt11 cDNA expression library obtained from the mouse T-cell line KKF. Finally, we identified Fyn as a molecule that binds to the WASP N-terminal domain and demonstrated that the interaction between endogenous WASP and Fyn is interfered by the over-expression of WASP N-terminal domain and anti-WASP-EVH1 scFv intrabodies in T cells derived from these Tg mice.

Methods

Preparation of T cells

T cells were isolated from the spleens of WASP15 Tg mice (15), anti-WASP-EVH1 scFv Tg mice (16) and age-matched C57BL/6 mice and were purified by negative selection using microbeads conjugated to mouse CD45R (B220) antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) using the autoMACSTM system (Miltenyi Biotec) according to the manufacturer’s instructions. The purity of the resulting T-cell population was >80% by FACS analysis with FITC-conjugated anti-mouse CD3 antibody (BioLegend, San Diego, CA, USA).

Quantitative real-time PCR

Purified T cells (1 × 10⁷ cells) were seeded in 100-mm plastic dishes pre-coated with 20 μg ml⁻¹ anti-CD3ε antibody (145-2C11; BioLegend) and cultured at 37°C in RPMI 1640 medium containing 10% FCS for 14 h. RNA from the stimulated T cells was isolated using the SV total RNA isolation medium containing 10% FCS for 14 h. RNA from the stimulated T cells was isolated using the ReverTra Ace-system (Promega, Madison, WI, USA). cDNA was obtained using the SV total RNA isolation medium containing 10% FCS for 14 h. RNA from the stimulated T cells was isolated using the SV total RNA isolation system (Promega, Madison, WI, USA). cDNA was obtained using the ReverTra Ace-α™ first strand cDNA synthesis kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions and amplified with the LightCycler TaqMan Master kit (Roche Diagnostics, Mannheim, Germany).

Real-time PCR for mouse IL-2 was performed in a LightCycler 1.5 (Roche) using Universal ProbeLibrary probe #15 (Roche) and IL-2 primers (forward, 5`-GCTGGTATGGACC-TACAGGA-3`; reverse, 5`-ATCTGGGGAGTTCTAGGTT-3`). Mouse hypoxanthine phosphoribosyltransferase (HPRT) was employed as a relative standard, using Universal ProbeLibrary probe #22 (Roche) and HPRT primers (forward, 5`-TGATAATCCATTCTCTGACTGTA-3`; reverse, 5`-AAGACATTCTTTCCAGTAAAGTTG-3`).

IL-2 ELISA

Purified T cells (1 × 10⁶ cells) were seeded in 48-well tissue culture plates pre-coated with anti-CD3ε antibody and cultured at 37°C in RPMI 1640 medium containing 10% FCS for 24 h. The IL-2 levels in culture supernatants were quantified in triplicate with the ELISA MAX™ Set Deluxe (BioLegend) according to the manufacturer’s instructions.

Expression cloning and phage spot assay

WASP N-terminal domain-binding protein was isolated by expression cloning from the murine pre-T-cell line KKF cDNA library inserted into the λgt11 phage vector (17) using histidine (His)-tagged WASP15 as probe. Phage solutions with or without cDNA encoding the WASP N-terminal domain-binding protein were diluted and spotted onto agarose plates containing Escherichia coli. After a 3-h incubation at 42°C, isopropyl-β-D-galactopyranoside (IPTG)-treated nitrocellulose membranes were overlaid on the agarose plates for 3 h at 37°C to induce synthesis of the β-galactosidase fusion proteins. The membranes were blocked with Tris buffered saline with Tween 20 (TBST) buffer (10 mM Tris–HCl, pH 8.0, 0.15 M NaCl and 0.05% Tween 20) containing 5% w/v non-fat dry milk and incubated with probe proteins at 4°C overnight. Binding was detected by sequential incubation of membranes with anti-His-tag antibody (MBL, Nagoya, Japan) and alkaline phosphatase-conjugated anti-rabbit Igs (Dako, Glostrup, Denmark), followed by incubation in alkaline phosphatase reaction solution [0.5 mM MgCl₂ and 25 mM Na₂CO₃ (pH 9.8), containing 0.4 mM of nitroblue tetrazolium and 0.4 mM 5-bromo-4-chloro-3-indolylphosphate-p-toluidine salt (Nacalai Tesque, Kyoto, Japan)].

Construction of His and glutathione-S-transferase fusion proteins

cDNA fragments for WASP15 and glutathione-S-transferase (GST) were subcloned into the Ndel and Xhol sites of the pET-23a vector (Novagen, Madison, WI, USA). WASP15-His and GST-His fusion proteins were produced in BL21 E. coli cells and purified by Ni-sepharose 6 Fast Flow affinity chromatography according to the manufacturer’s instructions (GE Healthcare, Buckinghamshire, England). The GST-Fyn-SH3 and GST-Fyn-SH2 fusion proteins have been described elsewhere (18).

Expression vector construction

Hemagglutinin (HA) double-tagged vector, D-HA-pRC/CMV and pCAGGS-MCS mammalian expression vectors were described previously (19, 20). cDNA fragments for mouse Fyn and WASP full-length proteins were subcloned into the Nol site of the D-HA-pRC/CMV and pCAGGS-MCS vectors, respectively. T7-tagged WASP deletion mutant, T7-WASP15 and Myc-tagged anti-WASP-scFv constructs were described previously (15, 16).

Transient transfection

NIH3T3 cells were maintained in DMEM supplemented with 10% FCS, 50 U ml⁻¹ streptomycin and 4 mM l-glutamine. NIH3T3 cells were adjusted to a density of 1 × 10⁶ cells per 100 mm dish and were transfected with 10 μg plasmid DNA using the FuGENE 6 Transfection Reagent (Roche Diagnostics).

His and GST pull-down assay

Purified T cells from wild-type mice were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitor cocktail, Nacalai Tesque) on ice for 1 h. Cell lysates were centrifuged at 10 000 × g for 10 min at 4°C, and the supernatants were incubated with
GST-His or WASP15-His fusion protein at 4°C overnight. The protein complexes were pulled down with anti-His tag beads (His Tagged Protein Purification Kit; MBL) according to the manufacturer’s instructions, lysed with SDS sample buffer and immuno-blotted with anti-Fyn pAb, anti-Lck pAb, anti-WASP-interacting protein (WIP) pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-His pAb (MBL).

In GST pull-down assay, purified T cells (2 × 10⁷ cells) were stimulated in anti-CD3ε antibody-coated 100-mm plastic dishes for 15 min at 37°C. Cells were collected, washed with cold PBS and then lysed with RIPA buffer on ice for 1 h. Cell lysates were centrifuged at 10 000 × g for 10 min at 4°C, and the supernatants were incubated with 100 µl of glutathione sepharose beads (GE Healthcare) for 1 h at 4°C to remove non-specifically bound proteins. The cleared cell lysates were incubated with glutathione sepharose beads bound to 50 µg GST fusion proteins at 4°C overnight. Beads were washed with PBS, lysed with SDS sample buffer and immunoblotted with anti-WASP antibody (Santa Cruz Biotechnology) and anti-GST pAb (MBL).

**Immunoprecipitation and western blot analysis**

At 48-h after transfection, NIH3T3 cells were washed twice with PBS and lysed with RIPA buffer on ice for 1 h. The lysates were centrifuged at 10 000 × g for 10 min at 4°C and the supernatants were incubated with 100 µl of streptavidin-conjugated agarose beads (Upstate, Lake Placid, NY, USA) for 1 h at 4°C to remove non-specifically bound proteins. The cleared lysates were incubated with 5 µg biotin-conjugated anti-HA mAb (MBL) on ice overnight and immunoprecipitated with 50 µl streptavidin-conjugated agarose beads.

Purified T cells (1 × 10⁶ cells) were lysed with RIPA buffer on ice for 1 h. The lysates were centrifuged at 10 000 × g for 10 min at 4°C and the supernatants were incubated with 100 µl of protein G-agarose (GE Healthcare) for 1 h at 4°C to remove non-specifically bound proteins. The cleared lysates were incubated with 5 µg anti-Fyn mAb or anti-WIP pAb (Santa Cruz Biotechnology) on ice overnight and immunoprecipitated with 50 µl protein G-agarose (GE Healthcare). Purified T cells with stimulation by anti-CD3ε antibody were lysed and incubated with 50 µl of agarose-conjugated anti-phosphotyrosine (p-Tyr) mAb (Santa Cruz Biotechnology) at 4°C overnight. Following five times PBS washes, immunocomplexes were re-suspended in SDS sample buffer and boiled for 10 min.

Cell lysates and immunocomplexes were separated by SDS–PAGE (12.5 or 15% gel) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked with TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% w/v non-fat dry milk for 1 h at room temperature and then incubated with anti-WASP pAb (raised against a synthetic peptide representing WASP residues 224–238) (Upstate), anti-Fyn pAb, anti-WIP pAb, anti-β-actin mAb (Santa Cruz Biotechnology), anti-T7 pAb, anti-Myc pAb (MBL), anti-phospho-c-jun N-terminal kinase (JNK), anti-JNK, anti-phospho-p44/42 mitogen-activated protein kinase (MAPK), anti-p44/42 MAPK, anti-phospho-p38, anti-p38 antibodies (Cell Signaling Technology, Danvers, MA, USA), anti-nuclear factor of activated T cells (NFAT) c2 pAb or anti-histone deacetylase 1 (HDAC1) pAb (Santa Cruz Biotechnology), followed by HRP-conjugated anti-rabbit, anti-goat or anti-mouse Igs (Dako). Immunoreactive proteins were detected by the ECL reagent (GE Healthcare).

**Subcellular protein extraction**

Cell extracts of wild-type and WASP15 Tg T cells were fractionated by the ProteoExtract™ Subcellular Proteome Extraction Kit (Calbiochem, San Diego, CA, USA) according to the manufacturer’s instructions.

**Statistical analysis**

Statistical significance was assessed using Student’s t-test (Statistica 03J; StatSoft, Tulsa, OK, USA). The differences were considered significant when P-values were <0.01.

**Results**

**IL-2 production induced by TCR stimulation is inhibited by over-expression of WASP N-terminal domain and anti-WASP-EVH1 scFv intrabodies**

To assess the effects of over-expression of WASP N-terminal domain (WASP15) and anti-WASP-EVH1 scFv on IL-2 transcription, quantitative real-time PCR was performed on RNA isolated from T cells of C57BL/6, WASP15 Tg and anti-WASP-EVH1 scFv Tg mice following TCR stimulation. In contrast to the measurable up-regulation of IL-2 gene transcription upon TCR stimulation in wild-type T cells, T cells from WASP15 Tg and anti-WASP-EVH1 scFv Tg mice showed only one-third or half of the levels of IL-2 transcription, respectively (Fig. 1A). Impairment of IL-2 secretion from these Tg T cells was confirmed by ELISA (Fig. 1B), suggesting that the WASP N-terminal region (including the EVH1 domain) is important for WASP function in IL-2 production following TCR stimulation.

**Activation of MAPK and NFAT pathway induced by TCR stimulation in WASP Tg T cells**

The MAPKs, such as JNK, p44/42 and p38 MAPK, play important roles in regulating IL-2 expression (21). To assess whether over-expression of WASP15 affects MAPK activation, the extent of phosphorylation of the MAPKs was compared between wild-type and WASP15 Tg T cells at various time points following TCR stimulation. Overall, the phosphorylation profiles of these MAPKs upon TCR stimulation were similar between wild-type and WASP15 Tg T cells (Fig. 2A).

Transcriptional activation of NFAT is required for TCR signaling-mediated synthesis of IL-2; activated NFAT translocates from the cytosol into the nucleus upon TCR stimulation (22, 23). To assess the effects of over-expression of WASP15 on nuclear translocation of NFATc2, a member of the NFAT family, T cells from wild-type and WASP15 Tg mice were fractionated into their subcellular compartments, and each fraction was analyzed by western blotting (WB) with anti-NFATc2 antibody. NFATc2 was detected in the cytosolic fraction of wild-type and WASP15 Tg T cells, regardless of the absence or presence of TCR stimulation (Fig. 2B, top panel). However, nuclear NFATc2 was detected only in wild-type T cells following TCR stimulation and was not detected in WASP15 Tg T cells in this assay (Fig. 2B, middle panel). Blotting with anti-HDAC1 pAb as a nuclear fraction marker was carried out to validate
subcellular fractionation and the protein amount loaded in each lane (Fig. 2B, bottom panel). These observations demonstrate that the WASP N-terminal domain is important for WASP function in IL-2 production mediated by nuclear translocation of NFATc2 in stimulated T cells.

Isolation of WASP N-terminal domain-binding protein

To elucidate the molecular mechanisms of WASP N-terminal domain participation in TCR signaling, we sought to isolate molecules that associate with WASP15 by screening of the λgt11 cDNA expression library obtained from the murine
pre-T-cell line KKF (24). After three rounds of screening against $1 \times 10^6$ phage clones, two positive clones were isolated. To confirm specific binding to WASP15, the positive phage clones WASP-binding protein (WASP-BP) #1 and #9, as well as the negative control phage clones #7 and #14, were diluted and used for a phage spot binding assay. Recombinant proteins derived from WASP-BP #1 and #9 were specifically associated with WASP15-His (Fig. 3A).

Sequences of the two isolated cDNA clones (#1 and #9) were identical and encoded a section of the Fyn tyrosine kinase (aa 20–198) containing the SH3 and SH2 domains of Fyn. The binding specificity of WASP N-terminal domain to Fyn was confirmed by an in vitro His pull-down assay. T-cell lysates from wild-type mice were incubated with GST-His or WASP15-His fusion proteins and pulled down with anti-His tag beads. WASP15-His selectively

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**Fig. 3.** In vitro binding assay by His and GST pull-down. (A) Phage spot-binding assay to the WASP N-terminal domain. One microliter of phage solutions of WASP-BP #1 and #9, as well as negative control clones #7 and #14, was spotted onto agarose plates containing *Escherichia coli*. Phage solutions in each lane were diluted as indicated. After protein induction on IPTG-treated nitrocellulose membranes, membranes were blocked with TBST containing 5% w/v non-fat dry milk and incubated with 1 μg ml$^{-1}$ of WASP15-His probe. Binding was detected by sequential induction of membranes with anti-His tag pAb and alkaline phosphatase-conjugated anti-rabbit antibody, followed by a color development reagent. (B) T-cell lysates from wild-type mice were incubated with GST-His or WASP15-His fusion proteins and pulled down with anti-His tag beads. Bound proteins were immunoblotted with anti-Fyn (a), anti-Lck (b), anti-WIP (c) or anti-His pAb (d). (C) Schematic representation of truncated Fyn. GST was fused with the N-terminus of each Fyn deletion mutant. (D) Purified T cells from wild-type mice spleen, without (−) or with (+) TCR stimulation, were lysed with RIPA buffer and incubated with GST, GST-Fyn SH3 and GST-Fyn SH2 non-covalently coupled to glutathione sepharose beads. Bound proteins were immunoblotted with anti-WASP (a) or anti-GST pAb (b). Immunoblots are representative of three independent experiments.
associated with Fyn but not Lck in T cells (Fig. 3B, a and b). WIP is known to bind to the WASP N-terminal EVH1 domain (25). Although WIP was not detected in our cDNA library screening, we examined the binding of WIP and WASP N-terminal domain in T-cell lysates. As shown in Fig. 3(B), c, the specific interaction between the WASP N-terminus and WIP was confirmed in this pull-down assay. The protein levels of GST-His and WASP15-His using this assay were comparable (Fig. 3B, d). These results suggest that the WASP N-terminal domain has an affinity to both Fyn and WIP in T cells.

**Binding of WASP to recombinant Fyn SH3**

To confirm the association of WASP and Fyn at the protein level, an in vitro binding assay was performed by GST pull-down. The SH3 and SH2 domains of Fyn were synthesized in E. coli cells fused with GST (Fig. 3C). T-cell lysates from wild-type mice with or without TCR stimulation by anti-CD3ε antibody were incubated with the GST fusion proteins and pulled down with glutathione sepharose beads. Regardless of TCR stimulation status, WASP specifically associated with GST-Fyn SH3 but not GST-Fyn SH2 in the pull-down assays (Fig. 3D, a). The protein levels of GST, GST-Fyn SH3 and GST-Fyn SH2 were comparable (Fig. 3D, b). These results indicate that WASP selectively associated with the SH3 but not the SH2 domain of Fyn.

**Binding between Fyn and WASP is inhibited by co-expression of WASP N-terminal domain and anti-WASP-EVH1 scFv in gene-transfected NIH3T3 cells**

To demonstrate the physiological interaction between the WASP N-terminal domain and Fyn, DNA constructs of full-length Fyn (Fyn-full), full-length WASP (WASP-full), truncated WASP (WASP15) and anti-WASP-EVH1 scFv were co-transfected into NIH3T3 cells (Fig. 4A). In the immunoprecipitation (IP) analysis, a strong interaction between Fyn and WASP was observed in NIH3T3 cells that were transfected with the Fyn-full and WASP-full constructs (Fig. 4B, a, lane 1). In contrast, when WASP15 was co-expressed in NIH3T3 cells, the interaction between Fyn and WASP was inhibited (Fig. 4B, a, lane 2), possibly by a competitive binding of WASP15 to Fyn. Furthermore, the interaction between Fyn and WASP was similarly inhibited by anti-WASP-EVH1 scFv in NIH3T3 cells (Fig. 4B, a, lane 3), probably through intrabody masking of the WASP N-terminal EVH1 domain. The amount of immunoprecipitated Fyn (Fig. 4B, b) and the binding of WASP15 and Fyn were confirmed in immunoblots with anti-T7 tag pAb (Fig. 4B, c, lane 2). The protein levels of WASP-full (Fig. 4B, d) were comparable among all transfected samples, and expression of anti-WASP-EVH1 scFv was confirmed by WB with anti-Myc tag antibody (Fig. 4B, e, lane 3). Taken together, these results strongly suggest that Fyn specifically binds to the WASP N-terminus.

**Interaction between WASP N-terminal domain and Fyn SH3 in T cells**

To confirm the specific binding of WASP to Fyn SH3 in T cells, T-cell lysates from wild-type, WASP15 Tg and anti-WASP-EVH1 scFv Tg mice were incubated with GST or GST-Fyn SH3 fusion proteins and pulled down with glutathione sepharose beads. In contrast to the strong binding of WASP to the GST-Fyn SH3 in wild-type T cells, their interactions were remarkably inhibited in WASP15 Tg and anti-WASP-EVH1 scFv Tg mouse T cells (Fig. 5A, a). The protein levels of GST and GST-Fyn SH3 were comparable in the assay (Fig. 5A, b). These results suggest that over-expression of WASP15 and anti-WASP-EVH1 scFv specifically interferes with the specific binding between WASP N-terminal domain and the SH3 domain of Fyn in T cells.

**Formation of Fyn–WASP–WIP complex in T cells**

To detect the binding of endogenous Fyn and WASP in T cells, T-cell lysates from wild-type, WASP15 Tg and anti-WASP-EVH1 scFv Tg mice were immunoprecipitated with an anti-
Fyn mAb, and immunocomplexes were immunoblotted with anti-WASP pAb. In the IP analysis, specific binding between endogenous Fyn and WASP was clearly detected in wild-type T cells (Fig. 5B, a). In contrast, the interaction between Fyn and WASP was severely inhibited in WASP15 Tg T cells (Fig. 5B, a). In addition, the competitive binding of the truncated WASP to Fyn was demonstrated by immunoblotting with anti-T7 tag pAb (Fig. 5B, b). The production of anti-WASP-EVH1 scFv intrabodies also inhibited the binding of Fyn and WASP (Fig. 5B, a). Fyn was equivalently immunoprecipitated in all mice T cells (Fig. 5B, c) and sufficient expression of anti-WASP-EVH1 scFv was confirmed by immunoblotting with anti-Myc tag pAb (Fig. 5B, d). Taken together, these observations strongly implicate the interaction between endogenous Fyn and WASP in T cells, possibly mediated by the binding of the Fyn SH3 and WASP N-terminal domains.

To assess the effects of over-expression of WASP15 and anti-WASP-EVH1 scFv on the interaction between Fyn and WASP.
endogenous WIP and WASP, T-cell lysates from wild-type, WASP15 Tg and anti-WASP-EVH1 scFv Tg mice were immunoprecipitated with anti-WIP antibody, and immunocomplexes were immunoblotted with anti-WASP pAb. In wild-type T cells, a strong binding between WIP and WASP was observed (Fig. 5C, a). In contrast, the interaction between WIP and WASP was inhibited in WASP15 Tg T cells (Fig. 5C, c), possibly by the competitive binding of WASP N-terminal domain to WIP, as demonstrated by immunoblotting with anti-T7 tag pAb (Fig. 5C, b). Expression of anti-WASP-EVH1 scFv also inhibited the binding of WIP and WASP (Fig. 5C, a), probably by the intrabody masking of the WIP-binding site in the WASP N-terminal EVH1 domain. Furthermore, to demonstrate the Fyn–WIP complex in TCR signaling, immunoprecipitates with anti-WIP antibody were immunoblotted with anti-Fyn pAb. The interaction of Fyn was clearly detected in wild-type T cells. However, by expression of WASP N-terminal domain and anti-WASP-EVH1 scFv, the binding of Fyn was inhibited in both Tg mice T cells (Fig. 5C, c). The inhibitory effects by these transgenes were similarly observed in Fyn–WASP and WIP–WASP interactions (Fig. 5B, a and Fig. 5C, a). WIP was equivalently immunoprecipitated in all mice T cells (Fig. 5C, c). Protein levels of endogenous WASP, Fyn and WIP were comparable in all mice T cells, so as the expression levels of β-actin (Fig. 5D). These results strongly suggest the existence of Fyn–WASP–WIP complex in TCR signaling.

Tyrosine phosphorylation of WIP is inhibited by over-expression of WASP N-terminal domain and anti-WASP-EVH1 scFv

To assess whether over-expression of WASP N-terminal domain and anti-WASP-EVH1 scFv affects tyrosine phosphorylation of WASP induced by TCR stimulation, the extent of TCR-induced tyrosine phosphorylation of WASP was compared between wild-type, WASP15 Tg and anti-WASP-EVH1 scFv Tg T cells by IP analysis. Tyrosine phosphorylation of WASP upon TCR stimulation was clearly detected in wild-type mice T cells, but it was markedly reduced in WASP15 Tg and anti-WASP-EVH1 scFv Tg mice T cells (Fig. 5E, a). In contrast, Fyn was equivalently tyrosine phosphorylated upon TCR stimulation among all mice T cells (Fig. 5E, b). These results suggest that over-expression of WASP N-terminal domain or anti-WASP-EVH1 scFv inhibits tyrosine phosphorylation of WASP by interfering the binding of Fyn to the WASP N-terminus but does not affect the activation of Fyn upon TCR stimulation. Taken together, these results strongly implicate that Fyn, WASP and WIP are closely associated in the complex to modulate transcriptional activation of IL-2 in response to TCR signaling.

Discussion

Here, we have demonstrated for the first time that the WASP N-terminal domain binds to not only WIP but also the SH3 domain of Fyn in mouse T cells. Inhibition of these interactions by over-expression of WASP15 and anti-WASP-EVH1 scFv resulted in impairment of IL-2 synthesis, which accompanied defective nuclear translocation of NFAT upon TCR stimulation. These observations strongly suggest that the interaction of Fyn, WASP and WIP plays a critical role in IL-2 production mediated by TCR signaling pathway.

Fyn is a member of the Src family kinase group of proteins, which is involved in the development, maintenance and activation of T cells. Fyn has a typical Src family kinase structure: an N-terminal unique domain, a Src homology 3 (SH3) domain, an SH2 domain, a tyrosine kinase domain and a C-terminal negative regulatory domain. The SH3 and SH2 domains of Fyn are involved in intra- and inter-molecular regulation through the mediation of protein–protein interactions via poly-proline and phosphorysine-specific interactions, respectively (26–28). The functional specificities of Fyn depend on the binding specificities of the SH3 and SH2 domains of Fyn to the target proteins (29).

Several lines of evidence suggest the possible interaction of Fyn and WASP in transformed monocyes and lymphocytes (14, 30, 31). In vitro binding assays implicated the C-terminal WASP PRR as a possible binding site for the Fyn SH3 domain (31). However, we demonstrated a physiological interaction between Fyn and WASP that was inhibited by over-expression of WASP15 and anti-WASP-EVH1 scFv intrabodies in gene-transfected NIH3T3 cells and Tg mouse T cells. These observations strongly support the hypothesis that Fyn specifically interacts with the WASP N-terminal region through its SH3 domain and phosphorylates WASP to modulate the WASP function in IL-2 production upon TCR stimulation.

WIP is identified as a binding partner of WASP and highly expressed in lymphoid tissues (25). WIP plays an important role in the recruitment of WASP to the immunological synapse after TCR ligation (32). WIP-binding site has already been determined within the N-terminal 170 amino acids of WASP (25). Although WIP was not isolated in our initial screening by the expression cloning, the specific binding of WIP and WASP in T cells was subsequently demonstrated in the in vitro binding assay (Figs. 3B and 5C). As shown in Fyn–WASP binding, the over-expression of WASP N-terminal domain and anti-WASP-EVH1 scFv intrabody strongly inhibited the binding of WIP and WASP in Tg mice T cells (Fig. 5C, a). de la Fuente et al. (33) demonstrated that expression levels of WASP, but not mRNA levels, were severely diminished in T cells from WIP knockout mice, suggesting that the binding of WIP and WASP is necessary for the stabilization of WASP. However, over-expression of WASP N-terminal domain and anti-WASP-EVH1 scFv intrabody, which strongly inhibits the binding of WIP and WASP, does not affect the expression of WASP, also Fyn and WIP in T cells (Fig. 5D).

In the present study, WIP–WASP–Fyn signaling complex was observed in wild-type mice T cells, but this complex formation was inhibited in WASP15 Tg and anti-WASP-EVH1 scFv Tg mice T cells (Fig. 5C, c). These observations strongly suggest the importance of complex formation of WIP–WASP–Fyn for the production of IL-2 in activated T cells.

Activation of MAPKs (JNK, p44/42, p38) and NFAT in response to TCR stimulation leads to induction of IL-2 synthesis (21–23). The MAPK phosphorylation profiles of wild-type and WASP15 Tg T cells upon TCR stimulation were similar to each other (Fig. 2A). Also, the profiles of Ca2+ influx after TCR stimulation were very similar between wild-type and WASP15 Tg mice T cells (data not shown). Nevertheless, over-expression of WASP15 strongly inhibited the nuclear
translocation of NAFTc2, as observed in T cells from wild-type mice (Fig. 2B). These results suggest that WASP may be involved in the signaling pathway after Ca²⁺ influx and regulate nuclear translocation of NFATc2. Calcineurin is a key enzyme that links Ca²⁺ signals to nuclear translocation of NFAT in T-cell activation (34). Several endogenous calcineurin inhibitors have been identified (35), including the protein family of regulators of calcineurin (RCAN, previously known as calcipressins or DSCR1), which interact with calcineurin and inhibit its phosphatase activity (36). Over-expression of WASP N-terminal domain may possibly inhibit the calcineurin activity either directly or indirectly through the modulation of these calcineurin inhibitors, resulting in impairment of NFAT nuclear translocation in WASP15 Tg mice T cells. Further investigations should be necessary to define the molecular mechanism of WASP in calcineurin–NFAT signaling pathway in T cells.

Badour et al. (31) demonstrated that phosphorylation of WASP tyrosine residue at position 291 in human, at position 293 in mouse, is induced by TCR stimulation and required for induction of NFAT translocation. TCR-induced translocation of phosphoseryl phosphorylation of WASP was diminished in WASP15 Tg and anti-WASP-EVH1 scFv Tg mice T cells (Fig. 5E), suggesting that interference of the binding between Fyn and endogenous WASP blocks the tyrosine phosphorylation of WASP by activated Fyn following TCR stimulation. The phosphorylated tyrosine residue and C-terminal PRR of WASP may possibly be targeted by another SH2 or SH3 domain-containing molecules, which transduce the TCR signaling to the nucleus. The identification of molecules downstream of the WIP–WASP–Fyn complex in the TCR signaling cascade will provide insight into the molecular mechanism for IL-2 synthesis in T cells.

As shown in Fig. 1(A) and (B), the inhibitory effects by expression of WASP15 and anti-WASP-EVH1 scFv were different in IL-2 synthesis upon TCR stimulation. Over-expressed WASP15 specifically binds to the Fyn SH3 domain and strongly inhibits the interaction between Fyn and endogenous WASP. Furthermore, the masking of the Fyn SH3 domain by WASP15 may interfere the interaction of Fyn to other PRR-containing signaling molecules, which have important roles in TCR signaling. In contrast, anti-WASP-EVH1 scFv intrabodies principally interfere the specific interaction between the Fyn SH3 domain and the WASP N-terminal domain by masking of this domain but do not affect the function of the Fyn SH3 domain itself. The difference in the magnitude of inhibition of IL-2 production may be explained by the difference in action mechanisms of the two interventions between WASP15 and anti-WASP-EVH1 scFv.

Two pharmacological agents, cyclosporin A and FK506, block IL-2 expression upon TCR stimulation. These inhibitors block the phosphatase activity of calcineurin and inhibit nuclear translocation of NFAT (37, 38). However, these inhibitors are not specific for NFAT proteins in the immune cells and cause detrimental side-effects, such as neurotoxicity and nephrotoxicity in clinical applications (39, 40). So, the search for drugs that specifically interfere or disrupt the interaction between the Fyn SH3 domain and the WASP N-terminal domain may result in a new type of immunosuppressive agents with fewer side-effects.

**Funding**

This work was supported in part by a program for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

**References**


A key role of Fyn–WASP complex in TCR signaling


