Overexpression of the Wiskott-Aldrich Syndrome Protein N-Terminal Domain in Transgenic Mice Inhibits T Cell Proliferative Responses Via TCR Signaling Without Affecting Cytoskeletal Rearrangements[1]

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Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by thrombocytopenia with small platelets, severe eczema, and recurrent infections due to defects in the immune system. The disease arises from mutations in the gene encoding the WAS protein (WASP), which plays a role as an adaptor molecule in signal transduction accompanied by cytoskeletal rearrangement in T cells. To investigate the functional domain of WASP, we developed transgenic mice overexpressing the WASP N-terminal region (exon 1–5) including the Ena/VASP homology 1 (pleckstrin homology/WASP homology 1) domain, in which the majority of mutations in WAS patients have been observed. WASP transgenic mice develop and grow normally under the specific pathogen-free environment, and showed normal lymphocyte development. However, proliferative responses and cytokine production induced by TCR stimulation were strongly inhibited in transgenic mice, whereas Ag receptor capping and actin polymerization were normal. These findings suggest that overexpressed Ena/VASP homology 1 (pleckstrin homology/WASP homology 1) domain of WASP inhibits the signaling from TCR without coupling of cytoskeletal rearrangement. WASP transgenic mice shown here could be valuable tools for further understanding the WASP-mediated processes. ***The Journal of Immunology,*** **2001, 167: 4701–4709.**

Wiskott-Aldrich syndrome (WAS)[4] is a rare X-linked recessive disorder, which is characterized by thrombocytopenia, eczema, combined immunodeficiency, and in severe cases susceptibility to lymphoreticular malignancy and autoimmune phenomena. The disease affects many hemopoietic cell lineages, including the humoral and cell-mediated immune systems and platelets (1, 2). The candidate gene presumed responsible for the disease has been successfully identified as that encoding the WAS protein (WASP) by positional cloning (3, 4).

The genomic structures of WASP and its homologous mouse WASP have been determined. Each gene contains 12 exons, nearly identical in size and composition, which span <10 kb of genomic DNA. WASP and mouse WASP share 86% amino acid identity deduced from cDNA sequence, and most studies have suggested that their expression is limited to the hemopoietic cell lineage that is consistent with the cell type affected in WAS patients (3–7).

WASP contains multiple domains that enable it to interact with different proteins. The WASP homolog, N-WASP, is expressed in a variety of tissues and, despite having only 50% amino acid homology with WASP, displays a virtually identical multiple-domain structure (8). A pleckstrin homology (PH) domain at the N-terminal region of WASP is a divergent protein module of ~100 amino acids found in many proteins involved in signal transduction. There is evidence that PH domains are capable of binding phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) and regulating protein interactions beneath the cell membrane (9–13) despite low amino acid sequence homology among the PH domains. A proline-rich region binds to the Src homology 3 domain of the adaptor Nck, Grb2 (14, 15), and of several kinases, including Fyn, Tec, Itk, and Btk (16–18). All these molecules are known to play significant roles in signal transduction after cell stimulation, indicating that WASP is involved in the process of intracellular signaling. In contrast, WASP can bind Cdc42, a Rho family protein involved in cytoskeletal organization (19), through its GTPase-binding domain (GBD) (20). In the WASP C-terminal region, verprolin- and cofilin-homologous regions are located (8). Verprolin is a yeast protein involved in the architecture of the actin cytoskeleton (21), and cofilin is an actin binding protein with pH-dependent actin depolymerizing activity (22, 23). Other cytoskeleton-associated protein sequence homologies were identified in the N- and C-terminal regions denoted as WASP homology domains 1 and 2 (WH1 and WH2), respectively (20). The WH1 and PH domains partially overlap, whereas the WH2 domain is observed in the verprolin homologous region. Currently the PH/WH1 domain is designated as the Ena/VASP homology 1 (EVH1) domain based on structural analysis (24). The term PH/WH1 domain is parenthesized in this paper. Although their functions are not well known, the EVH1

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[4] Abbreviations used in this paper: WAS, Wiskott-Aldrich syndrome; WASP, WAS protein; Tg, transgenic; EVH1, Ena/VASP homology 1; PH, pleckstrin homology; WH, WASP homology; CYTD, cytochalasin D; GBD, GTPase-binding domain; WIP, WASP-interacting protein.

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(PH/WH1) and WH2 domains may be involved in localization and regulation of the actin cytoskeleton. These findings indicate that WASP may connect protein tyrosine kinase signaling pathways to cell motility driven by actin polymerization.

Studies on lymphocytes from WAS patients have provided insights into the potential functions of WASP. T cells from WAS patients show abnormal cell surface cytoarchitecture, documented by scanning electron microscopy and characterized by the paucity of microvillous surface projections found on normal T cells (25). Their proliferative responses to anti-CD3ε Ab stimulation are severely depressed or absent (26), associated with a decrease in IL-2 secretion. Moreover, Ag receptor stimulation of T cell lines from WAS patients resulted in aberrant actin polymerization and the formation of abnormal cell shapes (27).

WASP-deficient mice have recently been generated (28, 29). T cells from WASP-deficient mice showed impaired proliferation and IL-2 production induced by TCR stimulation. Furthermore, WASP-deficient T cells showed a marked reduction in Ag receptor capping and actin polymerization induced by TCR stimulation (28, 29).

To investigate further the functional domain of WASP, we have developed WASP transgenic (Tg) mice overexpressing the WASP N-terminal region (exon 1–5) including the EVH1 (PH/WH1) domain, in which the majority of missense and nonsense mutations have been observed. The relation between the function of EVH1 (PH/WH1) domain and the mechanisms of WAS disease caused by mutations in this region remains unknown. We also examined the effects of overexpressing truncated WASP in T cells. T cells from WASP Tg mice were impaired in proliferative response and IL-2 production induced by TCR stimulation, whereas Ag receptor capping and actin polymerization were normal. We demonstrate here that the EVH1 (PH/WH1) domain plays an important role in WASP function during TCR signaling.

Materials and Methods

Plasmid construction

A cDNA fragment for mouse WASP exon 1–5 (amino acids 1–171) was generated by PCR (5′ primer, 5′-CTCCGGGATCCGGCAGCATGAATAG TG-3′, and reverse primer, 5′-GTCCGGAATTCTCACTCCTCATTGA TTGGTGCTG-3′), digested with *Bam*HI-*Eco*RI, and cloned into a *Bam*HI/*Eco*RI site of pCR3 (Invitrogen, Carlsbad, CA) expression vector. The T7 tag (MASMTGGQQMG) (30, 31) was generated as follows: coding linker, containing a *Kpn*I site at the 5′ end of the linker (5′-CCATGGCCAGCA TGACCGGCGGCCAGCAGATGGGCG-3′), and noncoding linker, containing a *Bam*HI site at the end of the linker (5′-GATCCGCCCATCTGC TGGCCGCCGGTCATGCTGGCCATGGGTAC-3′), were annealed and inserted into a *Kpn*I/*Bam*HI site of pCR3/WASP exon 1–5 (to be generated fusion protein with T7 tag and WASP exon 1–5). This construct was designated as pCR/T7-WASP15. T7 tagged WASP exon 1–5 cDNA fragment was digested with *Hin*dIII-*Not*I from pCR/T7-WASP15 and subcloned into a *Hin*dIII/*Not*I site of pCAGGS-MCS expression vector (32, 33). This construct was designated as pCAG/T7-WASP15.

Generation of Tg mice

The transgene was excised from the plasmid vector with *Sal*I-*Nhe*I restriction enzymes, purified by agarose gel electrophoresis and the QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany), adjusted to a final concentration of 3 μg/ml, and microinjected into the fertilized egg pronuclei of C57BL/6J inbred strain mice. The injected eggs were then transferred into the oviducts of pseudopregnant female ICR mice. After birth, Tg mice were identified by Southern blot analysis of tail DNA, using a 600-bp fragment of T7-WASP15 cDNA as a probe.

Immunoblotting

Each tissue sample collected from control wild-type mice or WASP Tg mice was sonicated and lysed with SDS sample buffer. The tissue extracts were then boiled for 10 min. Proteins were separated by 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked with TBST buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) containing 5% w/v nonfat dry milk and incubated with anti-T7 Ab (Novagen, Madison, WI) or anti-WASP Ab (Upstate Biotechnology, Lake Placid, NY), followed by HRP-conjugated anti-mouse or anti-rabbit IgG (DAKO, Glostrup, Denmark). Immunoreactive proteins were detected by ECL (Amersham Pharmacia Biotech, Uppsala, Sweden).

Immunization and T cell proliferation assay

Wild-type and WASP Tg mice were immunized in both footpads and the base of the tail with 100 μg of OVA (Sigma, St. Louis, MO) in CFA (Difco, Detroit, MI) on day 0. On day 7, inguinal, popliteal, and aortic lymph nodes were removed, and single cell suspensions were prepared in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine, 50 μM 2-ME, 10 mM HEPES (all obtained from Life Technologies, Rockville, MD), and 1% normal mouse serum. To examine proliferation, 5×10^5 cells were added to each well of 96-well tissue culture plates and cultured with the indicated amount of OVA or control BSA at 37°C for 72 h, pulsed with 1 μCi of [^{3}H]thymidine for an additional 16 h, and then collected and scintillation counted. The maximal stimulation index was calculated as: cpm [^{3}H]thymidine incorporation OVA or control BSA-treated cells/cpm [^{3}H]thymidine incorporation untreated cells. For evaluation of cytokine production induced by OVA stimulation, 1×10^6 cells were cultured with the indicated amount of OVA in 48-well tissue culture plates. The cell culture supernatant was collected at 48 or 72 h. IL-2 and IL-4 in the supernatant were quantified by sandwich ELISA using OptEIA set for mouse cytokines (BD PharMingen, San Diego, CA) as suggested by the manufacturer.

Ag receptor stimulation

T cells were purified from WASP Tg mice spleen or age-matched wild-type control mice by magnetic sorting and removal of B cells with anti-mouse I-A^b Abs (M5/114.15.2; BD PharMingen) following by anti-rat IgG-coated magnetic beads (BioMag; PerSeptive Biosystems, Framingham, MA) using standard procedures. The purity of the resulting population exceeded 90% as confirmed by FACS analysis. For the proliferation assay, anti-CD3ε Abs (145-2C11; BD PharMingen) were adhered to 96-well tissue culture plates by incubating 10 μg/ml in PBS, pH 8.0, at 4°C for 6 h, after which the plates were washed with PBS, pH 7.2. Purified T cells were added to the Ab-coated wells (5×10^5 cells/well), and cultured at 37°C in RPMI 1640 medium containing 10% FBS. IL-2 (BD PharMingen) was added to indicated cultures at a concentration of 5 ng/ml. Furthermore, purified T cells were stimulated with PMA (10 ng/ml) and ionomycin (0.5 mM). B cells were purified from WASP Tg mice spleen or age-matched wild-type control mice by isolating B220$^+$ cells. The purity of the resulting population exceeded 90% as confirmed by FACS analysis. B cells were cultured in 96-well tissue culture plates (5×10^5 cells/well) in culture medium alone or in the presence of anti-mouse IgM Ab F(ab′)$_2$ (10 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA), anti-CD40Ab (10 μg/ml; BD PharMingen), or LPS (10 μg/ml; Sigma). Each stimulation was performed in the presence of exogenous IL-4 (2 ng/ml; PeproTech, Rocky Hill, NJ). After a 48-h culture, T and B cells were pulsed with 1 μCi of [^{3}H]thymidine for an additional 16 h, and then collected and scintillation counted. For evaluation of cytokine production, purified T cells from the spleen of wild-type or WASP Tg mice were cultured on anti-CD3ε Ab-coated 48-well tissue culture plates. The cell culture supernatant was collected at 24 or 48 h. IL-2, IL-4, and IL-5 in the supernatant were quantified by sandwich ELISA using OptEIA set for mouse cytokines (BD PharMingen) as suggested by the manufacturer.

Peripheral blood analyses

Red blood cell, white blood cell, and platelet counts as well as hemoglobin content were determined from fresh blood samples of five wild-type (male/female) and five WASP Tg mice (male/female) by measurement on a Coulter counter. Mice analyzed were 11 wk old.

FACS analyses

Single-cell suspensions of lymphoid cells were prepared and stained with Abs following standard procedures. Abs directed to CD3, CD4, CD8, CD28, IgM, or B220 (PE or FITC conjugated; BD PharMingen), were used to stain the cells.

T cell capping

A single-cell suspension of splenic cells from wild-type or WASP Tg mice was prepared. The cells were incubated in IMDM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME (all obtained from Life Technologies), containing 5 μg/ml biotin-conjugated anti-CD3ε Ab (BD PharMingen), either at 37°C or 4°C for 40 min. The

treated cells (5×10^4) were cytospun onto glass slides, fixed in 3.5% paraformaldehyde, soaked in 0.2 M lysine solution, blocked with PBS containing 1% BSA and 5 μg/ml Fc-block (anti-CD16/32 mAb; BD PharMingen), incubated with streptavidin-Alexa 546 (5 μg/ml; Molecular Probes, Eugene, OR), sealed with cover slips, and then immediately analyzed and photographed at ×80 using confocal microscopy (LSM510; Zeiss, Oberkochen, Germany). The rate of capping of unstimulated and stimulated T cells was determined by counting the number of caps in ~200 cells/experiment.

Staining of filamentous actin

For the analysis by flow cytometry of CD3-induced actin polymerization, purified T cells (5×10^6) from the spleens of wild-type or WASP Tg mice were stimulated in anti-CD3ϵ Ab-coated plastic culture dishes. The reactions were stopped by fixing the cells in 2% paraformaldehyde (Sigma) for 30 min at room temperature. After washes with PBS, cells were stained with rhodamine-phalloidin (Molecular Probes; 1/50 dilution in PBS/0.05% saponin (Sigma)) for 40 min at room temperature and washed before analysis by flow cytometry. Where indicated, cells were pretreated for 45 min at 37°C with 4 μmol/L cytochalasin D (CYTD; Sigma) and stimulated with anti-CD3ϵ Ab immediately afterward. CYTD is an agent that binds to the barbed end of actin filaments, inhibiting both the association and dissociation of subunits at that end (34).

Results

Generation of WASP Tg mice

The cDNA fragment encoding mWASP exon 1–5 (1–171 amino acids) containing the EVH1 (PH/WH1) domain, in which many mutations have been observed, was inserted into the multicloning

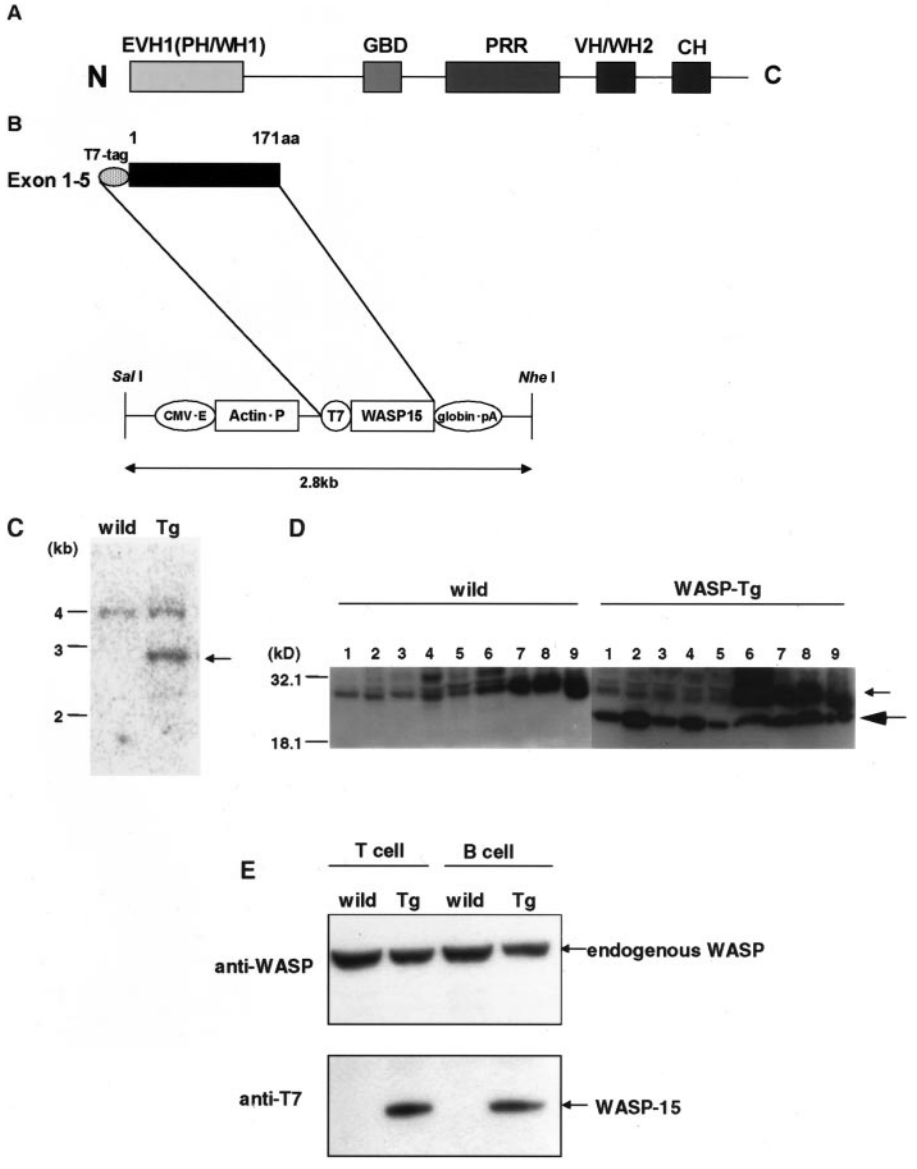


FIGURE 1. Construction and expression of the transgene in WASP Tg mice. *A*, Schematic representation of the WASP and the major functional domains. Shown are the EVH1 domain (PH/WH1), GBD, proline rich region (PRR), verprolin homology (VH)/WH2 domain, and cofilin homology (CH) domain. *B*, DNA construction for microinjection. CAG vector consists of expression units as shown. CMV · E, CMV enhancer; Actin · P, chicken β-actin promoter; globin · pA, rabbit β-globin poly(A). *C*, Southern blot analysis of tail DNA isolated from wild-type mice and WASP Tg mice. Using a 600-bp fragment of T7-mWASP-15 cDNA as probe, upon *Eco*RI digestion the inserted transgene into the mice genome was shown to 2.8 kb band (arrow). *D*, Western blot analysis of protein extracts of each tissue from control wild-type or WASP Tg mice. 1, Thymus; 2, heart; 3, lung; 4, liver; 5, kidney; 6, skin; 7, spleen; 8, lymph node; 9, bone marrow. The immunoblot was stained with anti-T7 mAb. The ~25-kDa band (large arrow) indicated the expression of T7-WASP-15 fusion protein. The ~28- to 32-kDa smear bands (small arrow) indicated second Ab cross-reactive IgG light chains. *E*, Western blot analysis of T and B cell lysates from spleens of control wild-type or WASP Tg mice. The equivalent of 5×10^6 cells was lysed and loaded in each lane. The immunoblots were probed with anti-WASP Ab (*top panel*) or anti-T7 Ab (*bottom panel*).

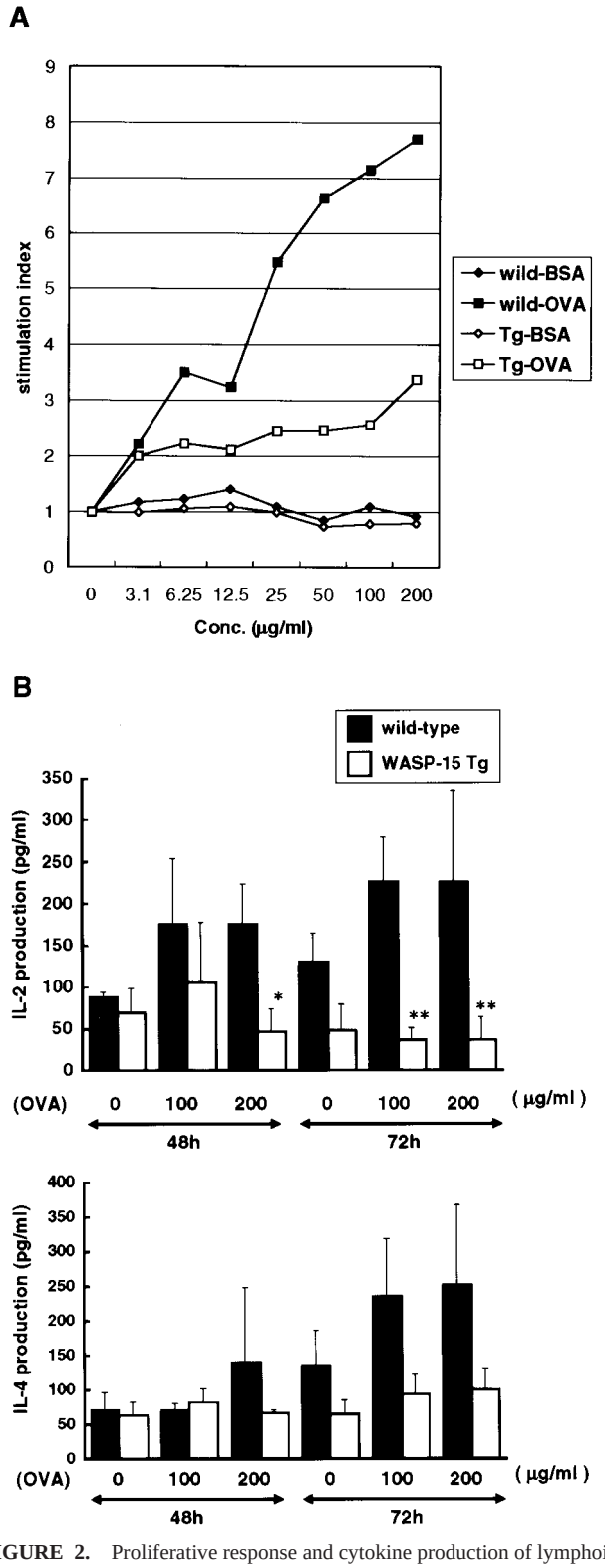


FIGURE 2. Proliferative response and cytokine production of lymphoid cells from wild-type and WASP Tg mice stimulated with OVA. Mice were immunized in both footpads and the base of the tail with 100 μg of OVA in CFA (day 0). Inguinal, popliteal, and periaortic lymph nodes were removed, and single cell suspensions were prepared in RPMI 1640 medium (day 7). *A*, Cells (5×10^5) were added to each well of 96-well tissue culture plates and cultured with the indicated amount of OVA or control BSA at 37°C for 72 h, pulsed with 1 μCi of [^{3}H]thymidine for an additional 16 h, and then collected and scintillation counted. Stimulation indices (see *Materials and Methods*) were calculated using the arithmetic mean values of triplicate cultures (SE values were <10% of means). *B*, Lymphoid cells (1×10^6) from wild-type (filled bars) and WASP Tg mice (open bars) were cultured with the indicated amount of OVA in 48-well tissue culture plates. Each cell culture supernatant was collected at the end of 48 or 72 h. IL-2 and IL-4 in the supernatant were quantified by ELISA. Values represent means ± SE of triplicate cultures and are representative of three independent experiments. Statistical significance is indicated by $*$ ($p < 0.03$) and $**$ ($p < 0.01$). Both wild-type and WASP Tg mice used for these experiments were 8 wk old.

site of the pCAGGS-MCS expression vector. Because the T7 tag sequence was added to the N terminus of the WASP cDNA, the protein produced by the T7 tag sequence-coupled cDNA is easily detected by Western blot analysis using commercially available Abs (Fig. 1, *A* and *B*). The DNA construction was microinjected into fertilized eggs of C57BL/6J mice, and one founder Tg mouse was obtained by Southern blot analysis (Fig. 1*C*). The founder mouse was crossed with (C57BL/6J) wild-type mice to propagate Tg mice. The inheritance pattern of the transgene in the offspring showed a normal Mendelian transmission. This Tg line was designated as WASP-15 Tg.

To detect expression of T7-tagged truncated WASP (WASP-15), we performed Western blot analysis with anti-T7 mAb. In most tissues of the WASP-15 Tg mice, high expression of WASP-15 was detected (Fig. 1*D*). We confirmed that the pCAGGS-MCS expression vector was effective for overexpression of target genes in Tg mice. In total we have obtained five Tg founders carrying the T7-tagged WASP-15 expression vector. In three of these five lines, the same levels of expression of WASP-15 were detected (data not shown).

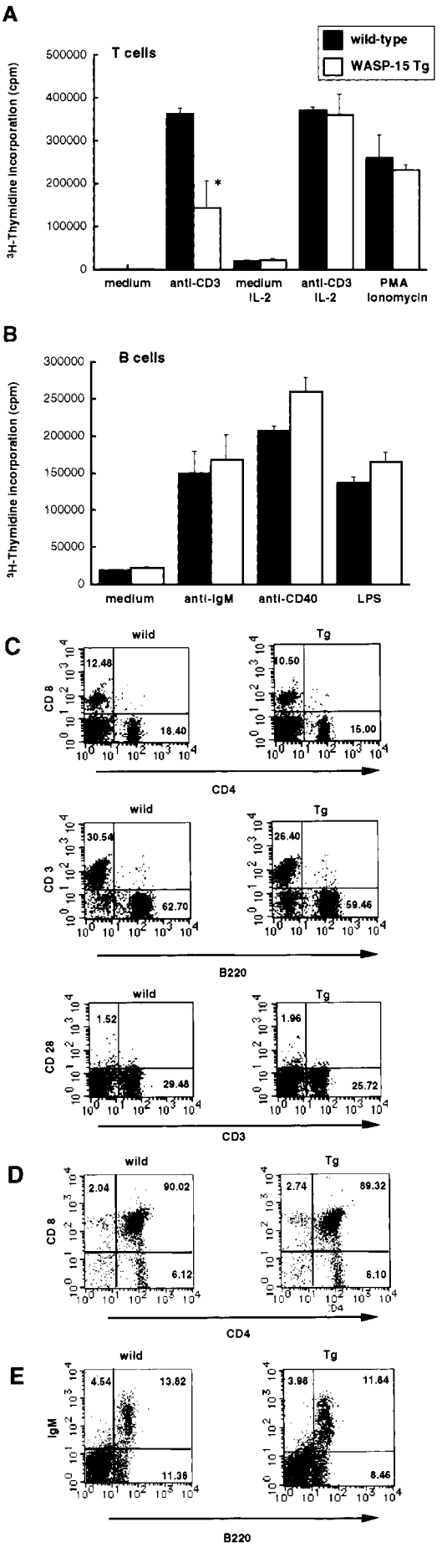
The WASP-15 Tg mice were normal in appearance, at least under maintenance in a specific pathogen-free environment. Unlike WAS patients, the WASP-15 Tg mice did not develop eczema. We looked specifically at the effects of the overexpressed WASP-15 on hemopoietic cell functions. Sufficient expression of WASP-15 was detected in WASP-15 Tg T cells (Fig. 1*E*). In addition, the expression level of endogenous WASP was almost the same between wild-type and WASP-15 Tg mice (Fig. 1*E*).

Lymphoid cells from WASP Tg mice immunized with OVA are impaired in proliferative response and cytokine production induced by secondary OVA stimulation

To assess the effects of the overexpressed WASP-15 on lymphoid cells, we examined the proliferative response to specific Ag. Wild-type and WASP-15 Tg mice were immunized in both footpads and tail base with OVA-CFA. After 7 days, inguinal, popliteal, and periaortic lymph nodes were removed, and single cell suspensions were cultured with OVA or control BSA for 48 h. As a result, lymphoid cells from WASP-15 Tg mice were impaired in proliferative response to the secondary OVA stimulation (Fig. 2*A*). To evaluate cytokine production induced by specific Ag, single cell suspensions from lymph nodes of wild-type and WASP-15 Tg mice immunized with OVA were cultured with OVA for 48 or 72 h, and cytokines in the culture supernatant were determined by ELISA. As shown in Fig. 2*B*, lymphoid cells from WASP-15 Tg mice were impaired in IL-2 and IL-4 production induced by the secondary OVA stimulation. These findings suggest that the impaired immune responses are caused by overexpressed WASP-15.

Impaired Ag receptor-induced proliferation in WASP Tg T cells, but not B cells

To assess the effects of the overexpressed WASP-15 on T cell function, we examined the proliferative response to stimulation with anti-CD3ϵ Ab (2c11). T cells purified from the spleens of WASP-15 Tg or wild-type mice were stimulated with anti-CD3ϵ



Ab immobilized on tissue culture wells. In contrast with wild type, T cells from WASP-15 Tg mice were impaired in their proliferative response to anti-CD3ϵ Ab stimulation (Fig. 3*A*). However, addition of exogenous IL-2 restored the normal proliferative response to anti-CD3ϵ Ab stimulation in WASP-15 Tg T cells. Furthermore, WASP-15 Tg T cells proliferated normally in response to PMA and ionomycin, which bypasses Ag receptor engagement (Fig. 3*A*). These findings indicate that WASP is required in signaling via the TCR, but not the IL-2R.

To assess the effects of the overexpressed WASP-15 on B cell function, we examined the proliferative response to Ag receptor stimulation. B cells from the spleens of WASP-15 Tg mice or wild-type mice were cultured with anti-IgM Ab $F(ab')_2$ in the presence of exogenous IL-4. The proliferative response to anti-IgM Ab $F(ab')_2$ was equivalent between wild-type and WASP-15 Tg B cells (Fig. 3*B*). Furthermore, the proliferative response to anti-CD40 Ab and LPS was also normal in WASP-15 Tg B cells (Fig. 3*B*). Therefore, in contrast to the result with T cells, these results indicate that WASP is not required for Ag receptor-induced proliferative response in B cells.

To confirm that there was no problem with the integration site of the transgene, we examined the other two Tg lines overexpressing WASP-15. T cells from the other two Tg lines were also impaired in proliferative response to stimulation with anti-CD3ϵ Ab (data not shown), confirming that there was no problem regarding the integration site.

Lymphoid development in WASP Tg mice

T cell development in the spleen can be followed by examining the expression pattern of the CD4 and CD8 surface Ags. The population of mature single-positive thymocytes (either $CD4^+CD8^-$ or $CD4^-CD8^+$) was almost the same between wild-type and WASP-15 Tg mice (Fig. 3*C*); likewise the expression pattern of CD3 was relatively equivalent. Furthermore, the percentages of splenic T and B lineage cell populations were normal (Fig. 3*C*). In addition, T lineage cell population in the thymus and B lineage cell population in the bone marrow were almost the same between wild-type and WASP-15 Tg mice (Fig. 3, *D* and *E*). Moreover, we observed relatively normal numbers of B1 cells in the peritoneum of WASP-15 Tg mice (data not shown). These findings indicate that the overexpressed WASP-15 does not have a marked effect on lymphocyte development.

FIGURE 3. Proliferative response of T and B cells induced by Ag receptor stimulation, and lymphoid development in wild-type and WASP Tg mice. *A,* T cell proliferation. Splenic T cells from WASP Tg (open bars) and age-matched wild-type (filled bars) mice were cultured in medium alone or in the presence of anti-CD3ϵ. Each stimulation was performed in the absence or presence of exogenous IL-2 as indicated. Signaling pathways bypassing TCR were assessed by stimulation with PMA and ionomycin. *B,* B cell proliferation. Splenic B cells from WASP Tg mice (open bars) and age-matched wild-type mice (filled bars) were cultured in medium alone or in the presence of anti-IgM Ab $F(ab')_2$, anti-CD40 Ab, or LPS. Each stimulation was performed in the presence of exogenous IL-4. In each experiment, cells were cultured for 48 h, pulsed with 1 μCi of [^{3}H]thymidine for an additional 16 h, and then collected and scintillation counted. Values represent means ± SE of triplicate cultures and are representative of three independent experiments. Statistical significance is indicated by $*$ ($p < 0.01$). *C–E,* FACS analyses of lymphocytes from wild-type and WASP Tg mice. Two-color flow cytometric analyses were performed on spleen (*C*), thymocytes (*D*), and bone marrow (*E*). Percentages of representative lymphoid populations are noted. The results shown are representative of at least three male mice of each type analyzed at the age of 8 wk.

Table I. *Peripheral blood analysis*[a]

Mice	Red Blood Cells ($\times 10^4/\mu$l)	White Blood Cells ($\times 10^2/\mu$l)	Platelets ($\times 10^4/\mu$l)	Hemoglobin (g/dl)
Wild-type (male)	964.4 ± 34.129	115.6 ± 22.233	103.4 ± 17.967	14.7 ± 0.37
(female)	946.2 ± 59.964	73.4 ± 9.685	95.6 ± 6.508*	14.4 ± 0.742
WASP Tg (male)	961.6 ± 44.168	111.4 ± 30.254	91.8 ± 7.378	14.7 ± 0.594
(female)	934.2 ± 64.107	76.8 ± 13.027	69.3 ± 10.241*	14.6 ± 0.618

[a] Data represent the mean ± SD of five animals in each group. *, Statistically significant difference, $p < 0.001$.

Reduced platelet numbers in female WASP Tg mice

The number of red blood cells and white blood cells in the peripheral blood of WASP-15 Tg mice was normal (Table I), but the number of platelets in the peripheral blood of female WASP-15 Tg mice was significantly reduced compared with wild-type and male WASP-15 Tg mice (Table I). We do not know why only the number of platelets in female WASP-15 Tg mice was reduced. However, we have not observed the reduction in platelet size or clinical signs of bleeding characteristic of WAS patients (data not shown). Furthermore, T cell proliferative response to stimulation with anti-CD3ϵ Ab were almost the same between male and female WASP-15 Tg mice (data not shown).

Purified T cells from WASP Tg mice are impaired in cytokine production induced by immobilized Anti-CD3 Ab

The overexpression of WASP-15 diminished the proliferative response to TCR-stimulation. To assess whether the overexpressed WASP-15 affects cytokine production induced by TCR stimulation, purified T cells from the spleens of wild-type or WASP-15 Tg mice were stimulated with immobilized anti-CD3ϵ Ab, and cytokines in the culture supernatant were determined by ELISA. T cells overexpressing WASP-15 were impaired in IL-2 production (Fig. 4*A*). In wild-type T cells, production of IL-4 and IL-5 was accelerated after IL-2 production, but in T cells overexpressing WASP-15, both IL-4 and IL-5 production were impaired because of diminished IL-2 production (Fig. 4, *B* and *C*). However, addition of exogenous IL-2 restored the normal production of IL-4 and IL-5 induced by anti-CD3ϵ Ab stimulation in WASP-15 Tg T cells (data not shown). These findings suggest that the overexpressed WASP-15 inhibits the signaling cascade of IL-2 production via TCR stimulation.

Ag receptor-induced capping and actin polymerization in WASP Tg T cells

The overexpression of WASP-15 diminished the proliferative response to TCR-stimulation. To assess whether the overexpressed WASP-15 affects TCR-induced capping, T cells from wild-type or WASP-15 Tg mice spleens were incubated in vitro with anti-CD3ϵ Ab, either at 37°C or 4°C (stimulated or unstimulated). The rate of Ag-receptor capping of T cells was not different between wild-type and WASP-15 Tg mice (Fig. 5*A*). In addition, we examined the ability of wild-type or WASP-15 Tg mice T cells to form new actin filaments upon anti-CD3ϵ engagement of the CD3/TCR complex. A polymerization of F-actin was determined by phalloidin binding changes detected in flow cytometry (Fig. 5*B*). In wild-type and WASP-15 Tg mice T cells, the fluorescence intensity increased until 30 min after stimulation, reflecting the normal kinetics associated with actin polymerization. However, CYTD-pretreated cells showed inhibition of normal actin polymerization. No differences between wild-type and WASP-15 Tg mice were found in fluorescence staining intensity when normal and CYTD-pretreated T cells were stimulated with anti-CD3ϵ Ab. These results indicate that the overexpressed WASP-15 does not affect the regulation of the cytoskeleton needed for the TCR-induced capping. Furthermore, these findings suggest that our WASP-15 directly or indirectly inhibits the signaling cascade of IL-2 production via TCR stimulation.

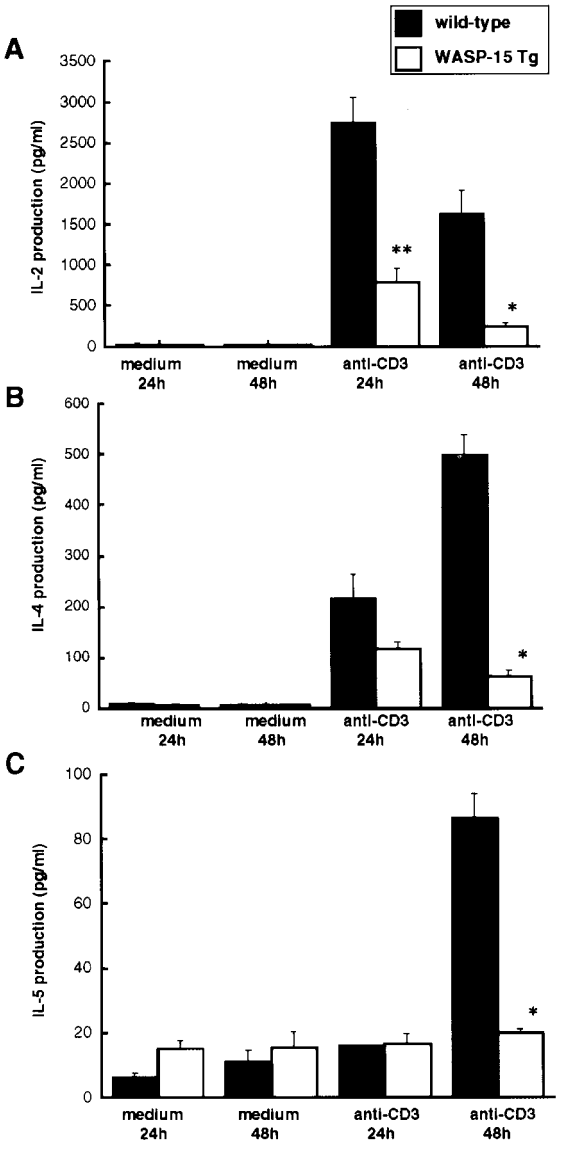


FIGURE 4. Cytokine production by wild-type and WASP Tg mice T cells stimulated with immobilized anti-CD3 Ab. Splenic T cells from WASP Tg (open bars) and age-matched wild-type (filled bars) mice were cultured in medium alone or in the presence of anti-CD3ϵ Ab. Each cell culture supernatant was collected at 24 or 48 h. IL-2 (*A*), IL-4 (*B*), and IL-5 (*C*) in the supernatant were quantified by ELISA. Values represent means ± SE of triplicate cultures and are representative of three independent experiments. Statistical significance is indicated by * ($p < 0.003$) and ** ($p = 0.014$). Both wild-type and WASP Tg mice used for these experiments were 8 wk old.

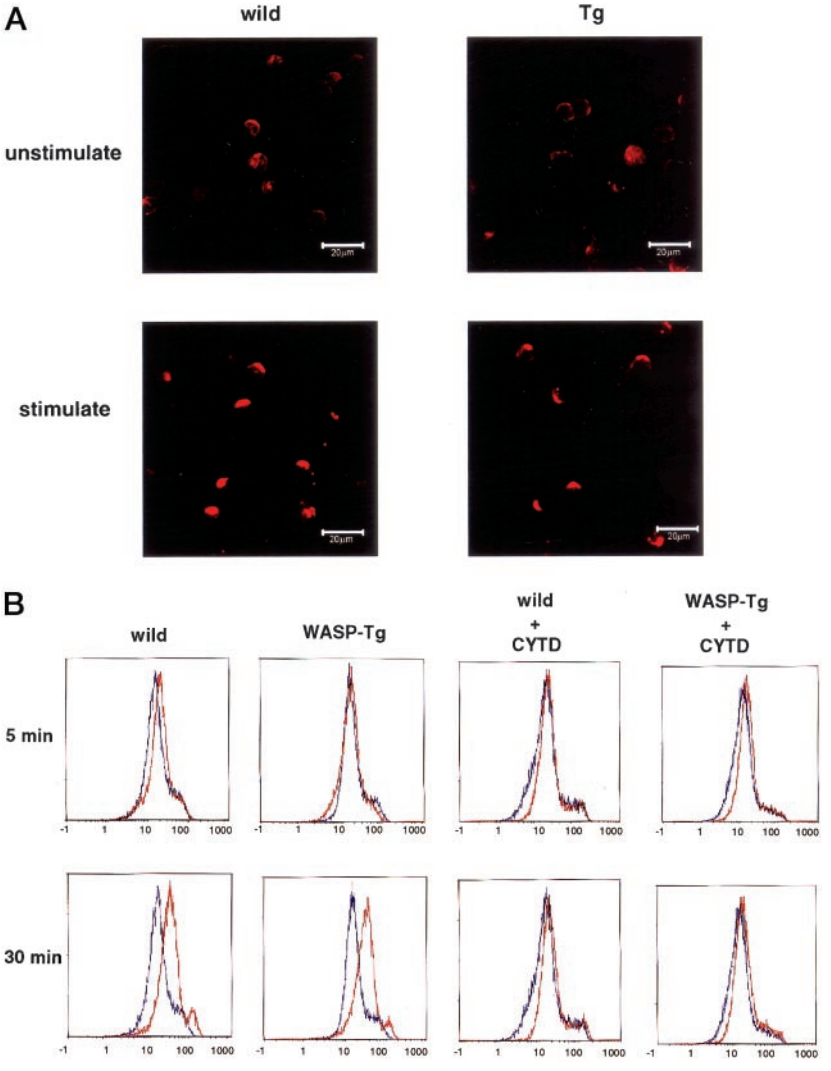


FIGURE 5. Ag receptor-induced capping and actin polymerization in wild-type and WASP Tg mice T cells. *A,* Single-cell suspensions of splenic cell from wild-type or WASP Tg mice were prepared. The cells were incubated with biotin-conjugated anti-CD3ϵ Ab, either at 4°C or 37°C for 40 min. The treated cells were cytospun onto glass slides, fixed, blocked with Fc-block (anti-CD16/32 mAb), and immunostained with streptavidin-Alexa 546. The rate of capping of unstimulated and stimulated T cells was determined by counting the number of caps in ~200 cells/experiment. Magnification = ×80. Both wild-type and WASP Tg mice used for this assay were 8 wk old. *B,* Purified T cells from the spleens of wild-type or WASP Tg mice were stimulated with immobilized anti-CD3ϵ Ab for indicated times, fixed, permeabilized, stained with rhodamine-phalloidin, and finally analyzed by flow cytometry. Fluorescence profiles of stimulated (red lines) and unstimulated (blue lines) cells are overlaid. Negative control cells were pretreated for 45 min at 37°C with 4 μmol/L CYTD and stimulated with anti-CD3ϵ Ab immediately afterward. One result representative of three independent experiments is shown. Both wild-type and WASP Tg mice used for these experiments were 8 wk old.

Discussion

To investigate further the functional domain of WASP, we have developed WASP-15 Tg mice overexpressing the EVH1 (PH/WH1) domain. In WASP-15 Tg T cells, proliferative response and cytokine production induced by TCR stimulation were strongly inhibited by overexpression of truncated WASP, whereas cytoskeletal rearrangement was normal. In the present study, we demonstrate that the EVH1 (PH/WH1) domain is important for WASP function during T cell activation.

Prominent symptoms attributed to WAS are immunodeficiency, eczema, and thrombocytopenia. In WASP-15 Tg mice, mild thrombocytopenia (decreasing platelet number) was observed only in female mice, whereas WASP-deficient mice showed mild thrombocytopenia and lymphopenia (decreasing numbers of blood lymphocytes) (28, 29) similar to WAS patients. In terms of eczema, neither WASP-15 Tg mice nor WASP-deficient mice displayed the phenotype when maintained in a specific pathogen-free environment. Moreover, lymphocyte development of both WASP-15 Tg mice and WASP-deficient mice was normal (28, 29).

T cell deficiency in WAS patients has been well demonstrated, and showed a correlation among human patients and the mutant mice. T cells from WAS patients and WASP-deficient mice failed to proliferate in response to Ag receptor stimulation (26, 28, 29). The same phenotype was found in WASP-15 Tg mice. Lymphoid cells from OVA-immunized WASP-15 Tg mice exhibited impaired proliferation induced by secondary OVA stimulation (Fig. 2*A*), and this proliferation defect was associated with diminished IL-2 and IL-4 production (Fig. 2*B*). Furthermore, purified WASP-15 Tg T cells were impaired in proliferation and IL-2, IL-4, and IL-5 production induced by anti-CD3ϵ Ab stimulation (Figs. 3*A* and 4, *A–C*). However, addition of exogenous IL-2 restored the normal proliferative response and IL-4 and IL-5 production induced by anti-CD3ϵ Ab stimulation in WASP-15 Tg T cells (Fig. 3*A* and data not shown). Moreover, WASP-15 Tg T cells proliferated normally in response to PMA and ionomycin, which bypasses Ag receptor engagement (Fig. 3*A*). These results suggested that WASP is required for signal transduction from the TCR, but not the IL-2R. WASP-15 Tg mice and WASP-deficient mice are clearly different in terms of the capacity of forming Ag receptor cap. Actin polymerization induced by TCR stimulation was impaired in WASP-deficient but not in WASP-15 Tg T cells. When incubated with anti-CD3ϵ Ab, WASP-15 Tg T cells were able to form Ag receptor caps (Fig. 5*A*), and actin filament polymerization was normal. This was determined through examining phalloidin-binding changes via flow cytometry (Fig. 5*B*). In contrast, WASP-deficient T cells showed a marked reduction in actin polymerization and Ag receptor capping after anti-CD3ϵ Ab stimulation (28, 29). The present results demonstrate that overexpression of the WASP N-terminal domain strongly inhibits TCR signal transduction in terms of IL-2 production, whereas the signaling process for the reorganization of the actin cytoskeleton remains intact.

It has been demonstrated that WASP binds to Cdc42, which is involved in cytoskeletal organization (19) through its GBD (20); WASP is also associated with the actin-related protein (Arp2/3) complex through its C-terminal region. The association between WASP and Arp2/3 complex activates the actin nucleation activity of the Arp2/3 complex (35–37). Because our truncated WASP lacks the normal GBD and the C-terminal region including a cofilin and verprolin homology domain, it cannot dominant negatively inhibit GBD and/or cofilin and verprolin homology domain functions. Such results support the idea that the WASP-GBD and C-terminal regions are important for the regulation of the actin cytoskeleton. In contrast, one would anticipate the overexpression of the EVH1 (PH/WH1) domain to hinder the correct association of key molecules for IL-2 production via TCR signaling. Thus, it is suggested that the WASP N-terminal domain may be important for the process of IL-2 production induced by TCR stimulation.

T cells from WASP-15 Tg mice were deficient in IL-2 production induced by TCR stimulation. The inability of WASP-deficient or WASP-15 Tg T cells to proliferate in response to TCR stimulation is similar to the defect observed in T cells from Vav-deficient mice (38, 39). It has been previously shown that Vav is a potent regulator of the IL-2 promoter, in particular NF-AT/AP-1-mediated gene transcription (40). Recently, WASP-interacting protein (WIP), a proline-rich protein of 503 amino acids, was isolated (41, 42). Furthermore, it has been observed that WIP and WASP interaction is important for Vav-mediated activation of NF-AT/AP-1 gene transcription induced by TCR stimulation (43). It is possible that the overexpressed WASP-15 inhibits WIP and endogenous WASP interaction because WASP-15 covers the WIP-interacting region encompassing WH1 and some neighboring proline residues. In addition to WIP, the overexpressed WASP-15 may inhibit interaction between endogenous WASP and other signaling molecules required for IL-2 production via TCR signaling. These findings support the hypothesis that WASP is a member of the LAT (linker for activation of T cells) complex, which consists of membrane-associated adaptor proteins (phospholipase C-γ1, Cbl, Vav, SLP-76, and Grb2) and plays an important role in T cell activation by coupling TCR ligation at the membrane to distal signaling cascades. The location of WASP-15 and specification of its associated molecules should be clarified.

We could not exclude the following possibility that the N-terminal domain of WASP may be involved in cytoskeletal rearrangement. Recently, it has been shown that the N-terminal WH1 domain of N-WASP is responsible for its recruitment to the site of actin polymerization with WIP (44). In this case, it is possible that the truncated WASP-WIP complex may directly or indirectly connect to other actin-regulating proteins and could induce actin polymerization and Ag receptor cap formation after TCR stimulation. However, in terms of IL-2 production, the correct association of key molecules for IL-2 production via TCR signaling may be inhibited by conformational changes in endogenous WASP.

Although WAS patients are apt to produce high IgE titer related to eczema, in WASP-15 Tg and WASP-deficient mice IgE titer was normal. Another feature of WASP-15 Tg mice was that B cells proliferated normally in response to anti-IgM Ab, anti-CD40 Ab, and LPS stimulation (Fig. 3*B*). Furthermore, WASP-15 Tg B cells produced normal Ab titers after OVA immunization (data not shown). B cells from WASP-deficient mice also proliferated normally after anti-IgM Ab stimulation (28, 29). Currently there is no clear explanation for the difference in B cell phenotype between WAS patients and such mutant mice.

In T and B cells, many intracellular proteins containing PH domain have been isolated, but their amino acid sequence homology is low among the PH domains. Overexpression of the WASP-15 led to only the T cell deficiency resulted in the defect of Ag receptor-mediated signaling, whereas B cell was normal. The similar phenotype has been observed in WASP-deficient mice, suggesting that the overexpressed WASP-15 specifically interferes with the signaling pathways relevant to endogenous WASP.

In conclusion, we were able to demonstrate that the EVH1 (PH/WH1) domain plays an important role in WASP function during TCR signaling. Therefore, WASP-15 Tg mice should be valuable tools for further understanding the in vivo function of WASP.

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