

T-cell receptor ligation causes Wiskott-Aldrich syndrome protein degradation and F-actin assembly downregulation

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Background: Wiskott-Aldrich syndrome protein (WASP) links T-cell receptor (TCR) signaling to the actin cytoskeleton. WASP is normally protected from degradation by the Ca^{++} -dependent protease calpain and by the proteasome because of its interaction with the WASP-interacting protein.

Objective: We investigated whether WASP is degraded after TCR ligation and whether its degradation downregulates F-actin assembly caused by TCR ligation.

Methods: Primary T cells, Jurkat T cells, and transfected 293T cells were used in immunoprecipitation experiments.

Intracellular F-actin content was measured in splenic T cells from wild-type, WASP-deficient, and c-Casitas B-lineage lymphoma (Cbl)-b-deficient mice by using flow cytometry. Calpeptin and MG-132 were used to inhibit calpain and the proteasome, respectively.

Results: A fraction of WASP in T cells was degraded by calpain and by the ubiquitin-proteasome pathway after TCR ligation. The Cbl-b and c-Cbl E3 ubiquitin ligases associated with WASP after TCR signaling and caused its ubiquitination. Inhibition of calpain and lack of Cbl-b resulted in a significantly more sustained increase in F-actin content after TCR ligation in wild-type T cells but not in WASP-deficient T cells.

Conclusion: TCR ligation causes WASP to be degraded by calpain and to be ubiquitinated by Cbl family E3 ligases, which targets it for destruction by the proteasome. WASP degradation might provide a mechanism for regulating WASP-dependent TCR-driven assembly of F-actin. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Wiskott-Aldrich syndrome, Wiskott-Aldrich syndrome protein, T-cell receptor, calpain, ubiquitination, proteasome, F-actin, Cbl family proteins

Abbreviations used

Arp:	Actin-related protein
Cbl:	Casitas B-lineage lymphoma
EVH1:	Ena-VASP homology domain 1
IS:	Immune synapse
TCR:	T-cell receptor
WAS:	Wiskott-Aldrich syndrome
WASP:	Wiskott-Aldrich syndrome protein
WIP:	WASP-interacting protein
WT:	Wild-type
ZAP-70:	ζ Chain-associated protein kinase of 70 kDa

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by variable immunodeficiency, eczema, and thrombocytopenia.¹ The gene for Wiskott-Aldrich syndrome protein (WASP) is mutated in patients with WAS and X-linked thrombocytopenia. WAS is located on Xp11.22-p11.23 and encodes a protein of 502 amino acids and approximately 60 kDa in molecular weight.^{2,3} WASP expression is restricted to hematopoietic cells.³

WASP has an N-terminal Ena/VASP homology domain 1 (EVH1) domain, a Cdc42/Rac GTPase-binding domain, a proline-rich domain, a G actin-binding verprolin homology (V) domain, a cofilin homology (C) domain, and a C-terminal acidic (A) segment.¹ The last 3 domains are located at the C-terminal end of WASP and are collectively referred to as the VCA domain. WASP interacts with WASP-interacting protein (WIP) through its EVH1 domain; with Cdc42-GTP through its GTPase-binding domain; with multiple SH3 domain-containing proteins, which include Nck, Grb2, and cortactin, through its proline-rich region; and with the actin-related protein (Arp) 2/3 complex that initiates actin polymerization through its VCA domain.⁴⁻⁶

WASP plays a critical role in T-cell activation and actin reorganization.⁷⁻⁹ T cells from patients with WAS and WASP^{-/-} mice are deficient in their ability to increase their F-actin content, secrete IL-2, and proliferate after T-cell receptor (TCR) ligation.¹⁰⁻¹² WASP exists in cells in a closed inactive conformation through intramolecular interactions that prevent the VCA domain from activating the Arp2/3 complex. Binding of Cdc42-GTP or of the SH3 domain of adaptor proteins, as well as phosphorylation of tyrosine (Y) at position 291, is thought to cause a conformational change in WASP, which allows the VCA domain to interact with and activate the Arp2/3 complex.^{5,13-15} The WASP-interacting protein (WIP) is expressed at high levels in lymphoid tissues. Most of WASP is associated with WIP in T cells.¹⁶ WIP binds through its C-terminal end to the EVH1 domain of WASP. WIP plays an important role in the recruitment of the WASP-WIP complex to ζ chain-associated protein kinase of 70 kDa (ZAP-70) and

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to the immunologic synapse after TCR ligation.¹⁷ More importantly, WIP stabilizes WASP in T cells. This is evidenced by the finding that WASP levels are significantly reduced in T cells from WIP^{-/-} mice and a WIP-deficient patient.^{16,18} Furthermore, most missense mutations in WASP that result in diminished WASP levels are localized to the WIP-binding EVH1 domain of WASP and disrupt the WASP-WIP interaction.^{19,20} Expression of the WASP-binding domain of WIP in these cells restores WASP levels close to normal.²¹ Treatment with calpain and proteasome inhibitors increases WASP protein levels in T cells from WIP^{-/-} mice and patients with WAS with missense mutations that disrupt WIP binding,¹⁶ indicating that WASP can be subject to degradation by calpain and the ubiquitin-proteasome pathway.

Unregulated activation of WASP is detrimental to many cell types, especially cells of the myeloid lineage. Three different mutations of WASP, namely L270P, S272P, and I294T, destroy the autoinhibitory conformation of WASP, resulting in a constitutively active protein, and cause X-linked neutropenia.²² The L270P and S272P mutations localize to the GTPase-binding domain,²³ whereas the I294T mutation is located close to the tyrosine residue Y291, which, when phosphorylated, results in the activation of WASP.²⁴ Knock-in mouse models mimicking the L270P and I294T mutations have been described.²⁵ T and B cells from these mice show a marked increase in F-actin levels but migrate normally in response to chemokines.

In this study we demonstrate that TCR ligation causes WASP to be degraded by calpain and by Casitas B-lineage lymphoma (Cbl)-mediated ubiquitination and proteasomal destruction. We demonstrate that WASP degradation provides a mechanism for downregulating TCR-driven assembly of F-actin.

METHODS

Cell lines and T cells

Jurkat T cells were obtained from the American Type Culture Collection and maintained in RPMI medium (Gibco, Carlsbad, Calif) supplemented with 10% FBS. 293T cells were a gift from Dr N. Ishii (Tohoku University, Sendai, Japan) and were maintained in Dulbecco modified Eagle medium (Gibco) supplemented with 10% FBS. Spleens from Cbl-b knockout (*Cbl-b*^{-/-}) mice and genetically matched wild-type (WT) shipping controls were a generous gift from Dr H. Gu, Columbia University. WASP-deficient mice were obtained from Dr Scott Snapper. Splenic T cells were isolated by using T-cell enrichment columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

Antibodies

Anti-WASP 5A5 mAb, which recognizes the epitope in the region corresponding to amino acids 146 to 265, was developed in our laboratory.²⁶ Anti-WASP rabbit polyclonal antibody K374 (a gift from Dr Ignacio Molina) is directed to the C-terminal 20 amino acids of WASP.¹⁶ Anti-phospho-WASP antibody, which recognized WASP phosphorylated on Y291, was purchased from Abcam (Cambridge, United Kingdom). Anti-ubiquitin mAb P4D1, anti-c-Cbl mAb A-9, and anti-Cbl-b mAb G-1 were from Santa Cruz Biotechnology (Santa Cruz, Calif). Anti-actin mAb and anti-FLAG mAb were from Sigma (St Louis, Mo). Anti-HA mAb was from Cell signaling (Danvers, Mass). Control rabbit IgG was from Upstate (Billerica, Mass).

TCR stimulation, immunoprecipitation, and Western blotting

TCR ligation was performed, as described previously.¹⁷ Briefly, T cells were incubated with 10 µg/mL anti-CD3 mAb UCHT1 (Calbiochem, San Diego, Calif) on ice for 20 minutes, followed by cross-linking with

15 µg/mL goat anti-mouse IgG (H+L; Caltag, Buckingham, United Kingdom) at 37°C for the indicated period. Cells were lysed in ice-cold lysis buffer containing 1% Triton X-100 and protease inhibitors. Cell lysates were clarified at 14,000g for 20 minutes at 4°C. For immunoprecipitation, cell lysates were precleared with protein G-Sepharose (GE Healthcare, Fairfield, Conn) for 2 hours and incubated overnight at 4°C with 4 µg of the indicated antibody preadsorbed onto protein G-Sepharose. Beads were washed 4 times with modified lysis buffer containing 0.2% Triton X-100. Bound proteins were eluted, run on 10% SDS-PAGE gels, and analyzed by means of Western blotting with the indicated antibodies followed by anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase and enhanced chemiluminescent detection (Amersham Life Sciences, Piscataway, NJ). Densitometry was performed with CS Analyzer version 2.08 software (ATTO Corporation, Tokyo, Japan) or ImageJ version 1.45 software.

Calpain and proteasome inhibition

The proteasome inhibitor MG132 and the calpain inhibitor calpeptin were purchased from Calbiochem. Cells were cultured with calpeptin (1 µmol/L) or MG132 (10 µmol/L) for 6 hours before anti-CD3 stimulation.

Expression vectors and transfection

Human pcDNA3.1-EGFP-hWASP-WT was a generous gift from Dr K. A. Siminovitch (University of Toronto, Toronto, Ontario, Canada). Control pAcGFP1-C1 vector was purchased from Clontech (Mountain View, Calif). Human pcDNA3.1-3xFLAG-c-Cbl-WT, pcDNA3.1-3xFLAG-Cbl-b-WT, and pcDNA3.1-HA-Ub were gifts from Drs N. Ishii and Y. Tanaka (Tohoku University).²⁷ Control p3xFLAG-CMV-14 vector was purchased from Sigma. 293T cells were transiently transfected with lipofectamine, as described previously,²⁷ and harvested 48 hours after transfection.

Determination of cellular F-actin content

Flow cytometric analysis of F-actin content was performed, as described previously.¹⁶ Briefly, mouse T cells were purified by using negative selection with the Pan T Cell Isolation Kit (Miltenyi Biotec) and then incubated for 6 hours with 1 µmol/L calpeptin. Cells were then washed and incubated with 10 µg/mL rat anti-mouse CD3 mAb (Serotec, Oxford, United Kingdom) for 30 minutes on ice. Cells were stimulated for the indicated times by cross-linking with goat anti-rat IgG (H+L) secondary antibody (Jackson ImmunoResearch, West Grove, Pa). Cells were fixed in 4% formaldehyde, washed, and permeabilized with the CytoFix/CytoPerm cell staining kit (BD Biosciences, San Jose, Calif). F-actin was stained with tetramethylrhodamine isothiocyanate-labeled phalloidin (Invitrogen, Carlsbad, Calif). F-actin content was analyzed by measuring the mean fluorescent intensity with FACS LSRFortessa (Becton Dickinson) and FlowJo (TreeStar, Ashland, Ore) software.

Statistical analysis

Statistical analysis was performed with the Student *t* test.

RESULTS

WASP is C-terminally truncated by calpain after TCR ligation

Purified peripheral blood T cells were stimulated with anti-CD3 mAb followed by cross-linking with secondary antibody and cell lysates were immunoblotted for WASP to investigate whether WASP is degraded after TCR ligation. Immunoblotting with mAb 5A5, which recognizes an epitope in the region corresponding to amino acids 146 to 265, revealed a 62- to 64-kDa band in unstimulated T cells (Fig 1, A), as previously observed.²⁸ Stimulation with anti-CD3 resulted in the appearance of a 55-kDa

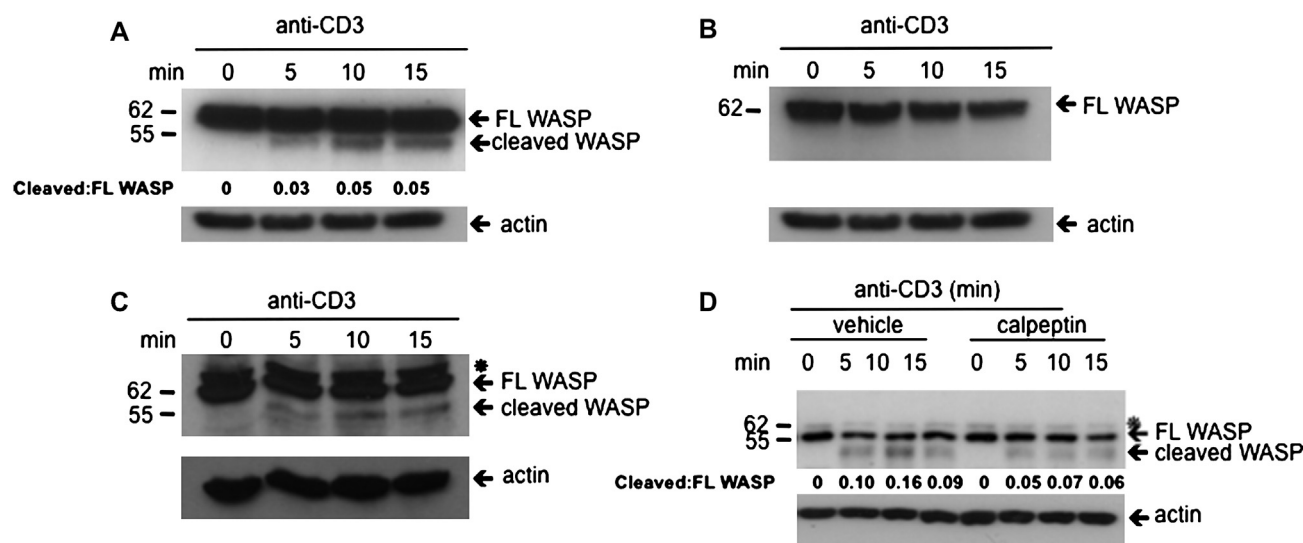


FIG 1. WASP is cleaved by calpain after TCR ligation. **A** and **B**, WASP immunoblot of peripheral blood T cells stimulated for 0 to 15 minutes with anti-CD3 mAb using mAb 5A5 (Fig 1, **A**) or polyclonal antibody K374 (Fig 1, **B**). **C**, WASP immunoblot of Jurkat T cells stimulated with anti-CD3 mAb using mAb 5A5. **D**, Effect of pretreatment for 6 hours with calpeptin on anti-CD3-driven WASP degradation in peripheral blood T cells. Lysates were immunoblotted with mAb 5A5. *Nonspecific band. The positions of molecular weight markers are indicated on the left in Fig 1, **A** to **D**. The ratio of cleaved WASP to full-length (FL) WASP in Fig 1, **A** and **D**, represents the mean of 5 experiments. Similar results were obtained in Fig 1, **A** to **D**, in 5 independent experiments.

WASP fragment at 5 minutes, which increased at 10 and 15 minutes after stimulation. Scanning densitometric analysis revealed that the intensity of the cleaved WASP band was approximately 5% that of the full-length WASP band at 10 and 15 minutes after stimulation. Similar results were obtained in Jurkat T cells (Fig 1, **C**).

Immunoblotting lysates of T cells with the rabbit polyclonal antibody K374 raised against the C-terminal 20 amino acids of WASP revealed the same 62- to 64-kDa band detected by using mAb 5A5 but did not detect the 55-kDa WASP fragment in anti-CD3-stimulated T cells that was detected by using mAb 5A5 (Fig 1, **B**). Similar results were obtained in Jurkat T cells (data not shown). This result indicates that the 55-kDa WASP fragment lacks the C-terminal VCA domains of WASP (amino acids 421-502) responsible for its actin-polymerizing activity.

Calpain cleaves WASP *in vitro*²⁹ and contributes to WASP degradation in WIP-deficient T cells.^{16,30} To examine whether calpain was responsible for the cleavage of WASP after TCR ligation, T cells were pretreated with the calpain inhibitor calpeptin for 6 hours, washed, and stimulated with anti-CD3 mAb for 5 minutes. Preincubation with calpeptin attenuated by approximately 50% the generation of the 55-kDa WASP fragment in response to anti-CD3 stimulation (Fig 1, **D**), strongly suggesting that calpain mediates the C-terminal truncation of WASP after TCR/CD3 ligation, at least in part.

WASP is ubiquitinated and degraded by the proteasome in T cells after TCR ligation

In the absence of WIP, WASP is degraded by the ubiquitin-proteasome pathway.^{16,30} To investigate whether WASP is a substrate for ubiquitination, we incubated *in vitro* transcribed and translated WASP with purified ubiquitin and ubiquitin-conjugating enzymes

(mixture of E1, E2, and E3 enzymes), and the reaction mixture was immunoblotted with anti-ubiquitin mAb. WASP was polyubiquitinated in the presence of ubiquitin and ubiquitin-conjugating enzymes, as indicated by an intense high-molecular-weight smear (Fig 2, **A**). Addition of the 26S proteasome fraction to the ubiquitination mixture resulted in marked attenuation of the ubiquitinated WASP smear. These results indicate that after TCR/CD3 ligation, WASP is subject to ubiquitination, which targets it for destruction by the proteasome.

We next examined whether WASP is ubiquitinated in T cells after TCR ligation. Fig 2, **B**, shows the appearance of polyubiquitinated WASP after anti-CD3 mAb stimulation of Jurkat T cells. To examine whether WASP ubiquitinated after TCR ligation is targeted for destruction by the proteasome, Jurkat T cells were pretreated with the proteasome inhibitor MG132 for 6 hours and then stimulated with anti-CD3 mAb for 10 minutes, and WASP immunoprecipitates were prepared from their lysates and probed for ubiquitin. Fig 2, **C**, shows that ubiquitinated WASP was weakly detectable in unstimulated Jurkat cells, but its levels increased after TCR/CD3 stimulation. Pretreatment with MG132 modestly increased the amounts of ubiquitinated WASP in unstimulated Jurkat cells and strongly increased the amounts of ubiquitinated WASP detected after TCR/CD3 ligation. These results indicate that WASP is ubiquitinated and degraded by the proteasome after TCR ligation.

The Cbl family proteins c-Cbl and Cbl-b associate with WASP after TCR ligation and act as E3 ubiquitin ligases for WASP

Members of the Cbl family of E3 ubiquitin ligases are negative regulators in TCR signaling.^{31,32} We tested the hypothesis that Cbl proteins might be involved in WASP ubiquitination. We first

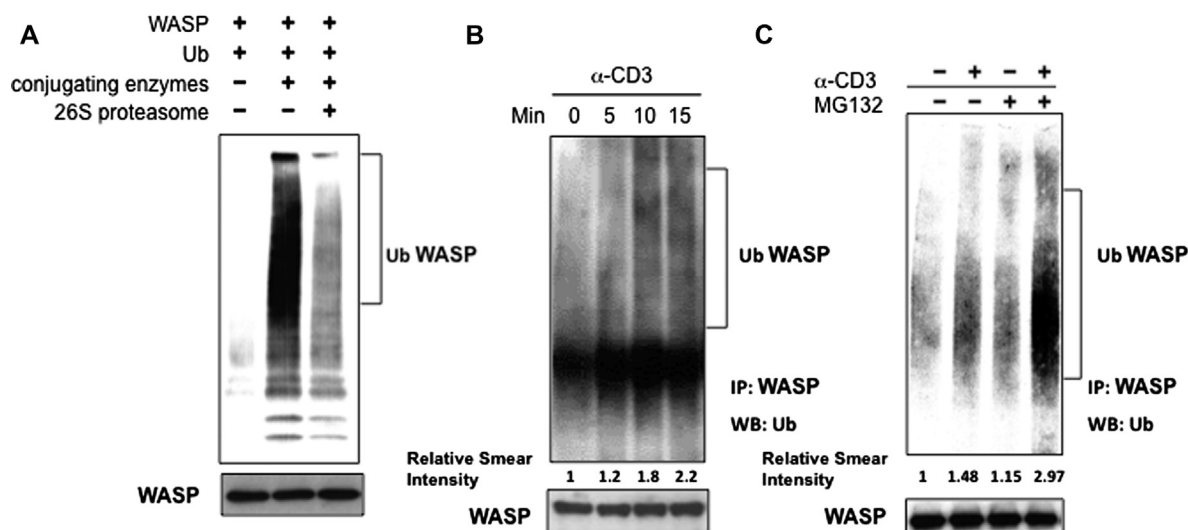


FIG 2. WASP is ubiquitinated and degraded by the 26S proteasome *in vitro* and in anti-CD3-stimulated Jurkat cells. **A**, Ubiquitination of *in vitro* translated purified WASP by ubiquitin-conjugating enzymes and its degradation by the 26S proteasome. Reaction mixtures were probed with anti-ubiquitin. **B**, Generation of ubiquitinated WASP in Jurkat T cells after stimulation with anti-CD3 mAb. WASP immunoprecipitates were probed with anti-ubiquitin mAb. Polyubiquitinated WASP appears as a smear. **C**, Protection of ubiquitinated WASP from degradation by the proteasome inhibitor MG132 in anti-CD3-stimulated Jurkat T cells. Similar results were obtained in Fig 2, A to C, in 4 independent experiments. The relative smear intensity in Fig 2, B and C, represents the mean of 4 experiments. IP, Immunoprecipitate; Ub, ubiquitin; WB, Western blot.

investigated whether Cbl proteins and WASP form a complex. WASP immunoprecipitates from Jurkat cell lysates were probed for c-Cbl and Cbl-b. c-Cbl, but not Cbl-b, coprecipitated weakly with WASP in unstimulated Jurkat T cells. TCR ligation increased the association of c-Cbl with WASP. It also induced the association of Cbl-b with WASP at 10 and 15 minutes after stimulation (Fig 3, A).

To investigate whether Cbl proteins act as E3 ubiquitin ligases for WASP, we transiently transfected 293T cells with plasmids coding for WT WASP, HA-tagged ubiquitin, FLAG-tagged c-Cbl, or FLAG-tagged Cbl-b. WASP coprecipitated with both c-Cbl and Cbl-b and was polyubiquitinated significantly more when co-transfected with ubiquitin, c-Cbl, and Cbl-b than with ubiquitin and empty vector (Fig 3, B).

To examine the role of Cbl-b in WASP ubiquitination after TCR ligation, we used purified T cells from spleens of *Cbl-b*^{-/-} mice. Ubiquitination of WASP after TCR ligation was reduced, although not completely abrogated, in T cells from *Cbl-b*^{-/-} mice (Fig 3, C), suggesting that WASP is a substrate for Cbl-b in antigen-stimulated T cells. We could not examine the role of c-Cbl on WASP ubiquitination after TCR ligation because we had no access to T cells from *c-Cbl*^{-/-} mice.

WASP degradation after TCR/CD3 ligation limits TCR/CD3-driven F-actin assembly in T cells

WASP is important for F-actin assembly in T cells.¹⁰ We examined whether WASP degradation after TCR/CD3 ligation regulates TCR/CD3-driven F-actin assembly. Purified T cells from WT and WASP-deficient mice were incubated for 6 hours with calpeptin or left untreated, washed and stimulated with anti-CD3 mAb, and cross-linked with a secondary antibody. The cells were then fixed, permeabilized, stained for F-actin with

fluorescein isothiocyanate-conjugated phalloidin, and analyzed by means of flow cytometry. As previously reported, WASP-deficient T cells had a lower F-actin content than WT T cells.¹⁶ TCR/CD3 ligation caused a parallel increase in F-actin levels in both WT and WASP-deficient T cells, which peaked at 5 minutes after stimulation and returned almost to baseline 10 minutes after stimulation. Pretreatment with calpeptin had no effect on F-actin content of the T cells at baseline or at 2 and 5 minutes after stimulation; however, it significantly increased the F-actin content of WT T cells at 10 minutes after anti-CD3 stimulation, maintaining it at almost the peak level achieved at 5 minutes after stimulation. In contrast, pretreatment with calpeptin had no effect on the F-actin content of WASP-deficient T cells 10 minutes after anti-CD3 stimulation. These results suggest that calpain-mediated WASP degradation limits the duration of F-actin assembly after TCR/CD3 ligation.

We next examined whether ubiquitination, which targets WASP for proteasomal degradation, regulates F-actin assembly after TCR/CD3 ligation. Because Cbl-b participates in WASP ubiquitination, we examined F-actin assembly in T cells deficient in Cbl-b. Baseline F-actin content and TCR-driven F-actin assembly were both significantly increased in T cells from c-Cbl-deficient mice compared with T cells from WT control animals (Fig 4, B). These results suggest that WASP degradation by ubiquitination regulates baseline and TCR-driven F-actin assembly.

DISCUSSION

Our results demonstrate that TCR ligation triggers the degradation of WASP by calpain-mediated cleavage and Cbl-mediated ubiquitination and subsequent proteasomal degradation. We present evidence that WASP degradation provides a mechanism for limiting the duration of TCR-driven assembly of F-actin.

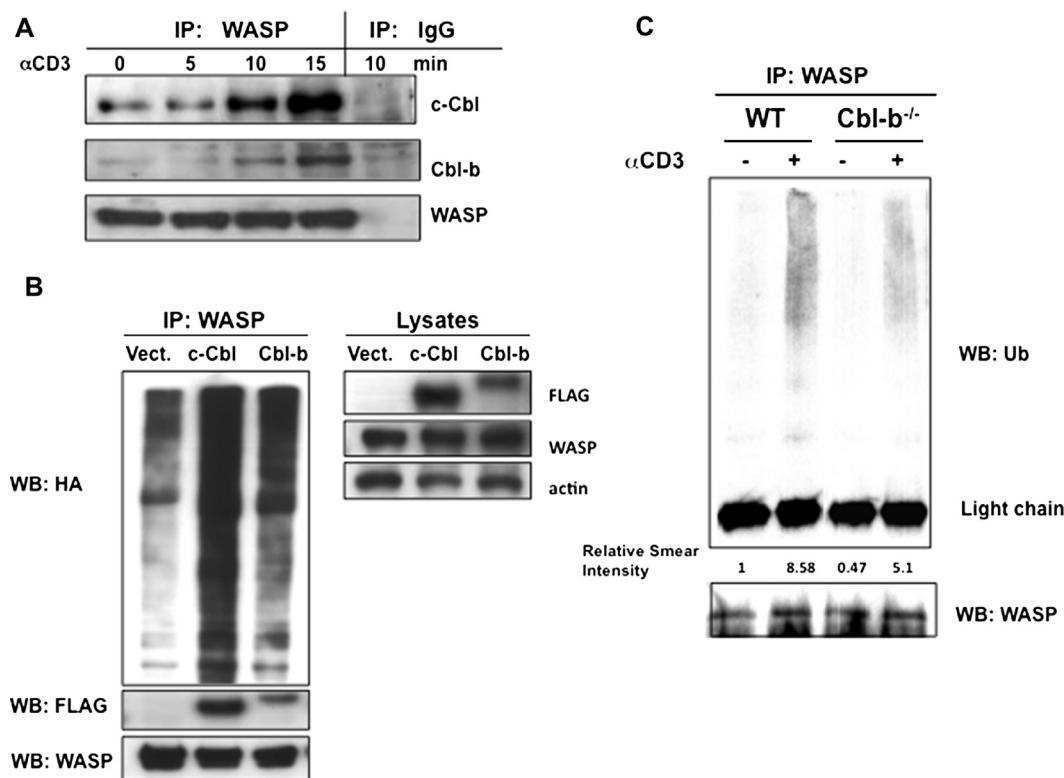


FIG 3. Cbl family E3 ubiquitin ligases associate with and ubiquitinate WASP after TCR ligation. **A**, Western blot analysis of WASP immunoprecipitates from anti-CD3-stimulated Jurkat T cells for c-Cbl and Cbl-b. IgG control antibody precipitates were prepared 10 minutes after anti-CD3 stimulation and used as controls. **B**, Ubiquitination of WASP in 293T cells transfected with WT WASP and HA-tagged ubiquitin plus either FLAG vector alone (*Vect.*), FLAG-tagged c-Cbl, or FLAG-tagged Cbl-b. In the *left panel* WASP immunoprecipitates were probed for HA, FLAG, and WASP. In the *right panel* total lysates were probed for FLAG-c-Cbl or FLAG-Cbl-b, WASP, and actin. **C**, WASP ubiquitination after TCR ligation in T cells from *Cbl-b*^{-/-} mice and WT control animals. WASP immunoprecipitates were probed for ubiquitin. Similar results were obtained in Fig 3, A to C, in 4 independent experiments. The relative smear intensity in Fig 3, C, represents the mean of 4 experiments. *IP*, Immunoprecipitate; *Ub*, ubiquitin; *WB*, Western blot.

TCR/CD3 ligation resulted in the degradation of a small fraction of WASP through calpain-mediated cleavage and the ubiquitin proteasome pathway. We estimated that approximately 5% of WASP is degraded after TCR/CD3 ligation. This is possibly an underestimate because the truncated 55-kDa WASP might be less stable than intact WASP. We could not detect a decrease in the levels of intact WASP in anti-CD3-activated T cells, probably because Western blotting is not sensitive enough to detect a small decrease in protein levels. We were unable to detect the cleaved, C-terminal, approximately 10-kDa fragment using an antibody to the C-terminus of WASP. This is most likely because such a small cleaved fragment would be rapidly degraded in the cell. Normally, WASP is protected from degradation by its partner, WIP.¹⁶ The conformational changes in WASP induced by TCR signaling, which involve a change from an inactive to an active form capable of activating the Arp2/3 complex and F-actin polymerization, possibly increases the susceptibility of WASP to calpain cleavage and to ubiquitination and proteasomal degradation. The observation that WASP is degraded by calpain after TCR ligation is consistent with previous observations that WASP can be degraded in platelet lysates by calpain²⁹ and that *in vitro* translated WASP is a substrate for calpain I and II.¹⁶ The increase in intracellular Ca⁺⁺ concentration that follows

TCR ligation could be the trigger for the Ca⁺⁺-dependent activation of calpain in anti-CD3-stimulated T cells.

Both c-Cbl and Cbl-b associated with WASP when overexpressed in 293T cells and acted as E3 ubiquitin ligases for WASP ubiquitination *in vitro*. More importantly, WASP ubiquitination after TCR ligation was impaired in Cbl-b-deficient T cells, implicating at least Cbl-b in WASP ubiquitination in T cells. Cbl family proteins act as negative regulators of TCR signaling by virtue of their ability to ubiquitinate LCK and ZAP-70,³³ which are upstream of WASP. Thus Cbl family members might regulate WASP activity indirectly by dampening TCR signaling upstream of WASP, as well as directly by ubiquitinating WASP and targeting it for degradation. Evidence has been presented that the activated WASP phosphorylated at Y291 is a target for ubiquitination.³⁴ We have also found that inhibition of the proteasome by MG132 increases the amount of tyrosine-phosphorylated WASP in anti-CD3-stimulated cells (see Fig E1 in this article's Online Repository at www.jacionline.org). This observation lends further support to the notion that activated WASP molecules are targets for degradation after TCR ligation.

It is not clear whether the interaction between WASP and c-Cbl and Cbl-b is direct or mediated by other proteins. It has been reported that c-Cbl associates with multiple proteins, which

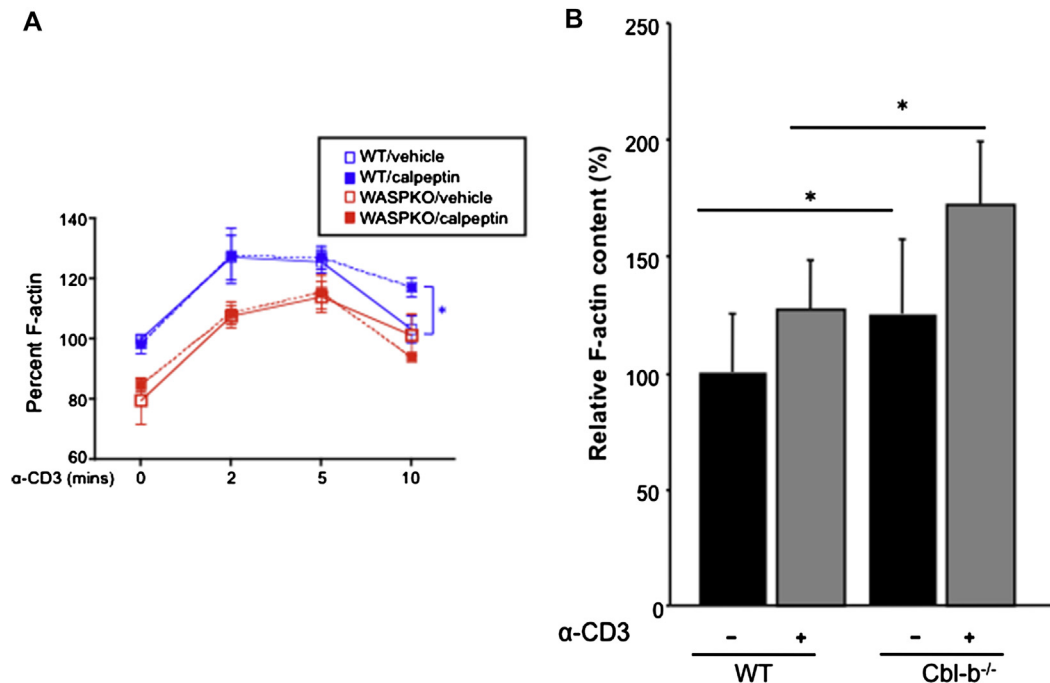


FIG 4. Effect of calpain inhibition and loss of Cbl on F-actin assembly in T cells. **A**, Effect of pretreatment of T cells from WT and WASP knockout mice with calpeptin on TCR-driven assembly of F-actin. Pretreatment with the vehicle dimethyl sulfoxide was used as a control. **B**, TCR-driven assembly of F-actin in T cells from *Cbl-b*^{-/-} mice and WT control animals. T cells were stimulated with anti-CD3 for 10 minutes. Results are expressed as a percentage of the baseline F-actin content in unstimulated WT T cells and represent the means $\pm$ SDs of 3 independent experiments. **P* < .01.

include tyrosine phosphorylated ZAP-70,³⁵ the adaptor proteins Nck³⁶ and Grb2³⁷ through their SH3 domains, CrkL through its SH2 domain,³⁸ Src tyrosine kinases through their SH3 domains,^{39,40} and Vav and the p85 regulatory subunit of phosphatidylinositol-3-OH kinase through their SH2 domains.^{41,42} Because these proteins are also reported to associate with WASP, its partner WIP, or both,^{17,43} an indirect association of Cbl with WASP cannot be ruled out. Alternatively, c-Cbl and Cbl-b could directly interact with an activated form of WASP, such as tyrosine-phosphorylated WASP. Indeed, while this manuscript was in preparation, it was shown that WASP phosphorylation at tyrosine 291 after TCR activation results in recruitment of Cbl-b.³⁴

Our data suggest that the degraded fraction of WASP includes activated WASP. This is supported by the observation that calpain inhibition and lack of the WASP-ubiquitinating E3 ligase Cbl-b resulted in more sustained F-actin assembly in WT T cells after TCR/CD3 ligation. The small fraction of WASP that is cleaved after TCR ligation could be important for F-actin polymerization because of its location close to the TCR. Indeed, we have shown previously that a fraction of WASP translocates together with a fraction of the TCR/CD3 complex to lipid rafts.¹⁷ It is also well known that a fraction of WASP colocalizes with TCR molecules in the immune synapse (IS).^{17,44,45} Cbl family molecules, which are also recruited to the IS, where they are activated by LCK and ZAP-70,^{46,47} could ubiquitinate WASP molecules recruited to the IS, targeting them for degradation. The IS is a dynamic structure that constantly undergoes protein kinase C θ -dependent dissolution and WASP/F-actin-dependent reformation of its peripheral supra-molecular activation complex.⁴⁵ Protein kinase C θ -dependent dissolution breaks the symmetry of the IS and allows T-cell motility.

WASP/F-actin-dependent reformation of the IS is important for the sustained signaling that is necessary for IL-2 production. We speculate that cycles of TCR-triggered recruitment and activation of WASP in the IS followed by local degradation of the activated WASP might be important for IS dynamics and T-cell function.

The observation that baseline F-actin content was increased in *Cbl-b*^{-/-} T cells, but not in calpeptin-treated T cells, suggests that under steady-state conditions, Cbl ubiquitination and proteasome degradation, but not calpain, degrade WASP molecules in activated T cells. The observation that calpain inhibition had no effect on F-actin assembly in WASP-deficient T cells indicates that calpain regulates F-actin assembly by targeting WASP for degradation. These results strongly suggest that degradation of activated WASP by calpain and by the ubiquitin/proteasome pathway provide an important homeostatic mechanism for terminating signaling to the cytoskeleton after TCR ligation. Furthermore, WASP mutants that are resistant to ubiquitination are associated with enhanced T-cell activation, supporting the notion that WASP degradation limits TCR activation.³⁴

Protein cleavage is used by prokaryotes and eukaryotes to activate or terminate signaling. Well-documented examples include the coagulation cascade, the complement activation cascade, degradation of the nuclear factor κ B inhibitor I κ B α , TNF receptor-associated factor 3, Argonaute, and voltage-gated calcium-channel proteins.⁴⁸⁻⁵³ Degradation of activated WASP might regulate receptor signaling to the cytoskeleton not only in T cells but also in other hematopoietic cells. Such a control mechanism would avoid the potential pathology observed in patients with mutations that cause sustained WASP activation and manifest as X-linked neutropenia.

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Key message

- TCR signaling causes WASP to be degraded by calpain and by Cbl-family members through ubiquitination and destruction by the proteasome, limiting TCR-driven assembly of F-actin.

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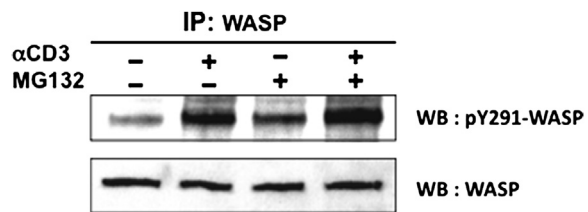


FIG E1. Tyrosine-phosphorylated WASP generated after TCR ligation is a target for proteasomal degradation. Effect of pretreatment of MG132 on the amount of tyrosine-phosphorylated WASP in anti-CD3-stimulated Jurkat T cells is shown. WASP immunoprecipitates were probed with anti-pY291-WASP antibody (Abcam). Similar results were obtained in 3 experiments. *IP*, Immunoprecipitate; *WB*, Western blot.