Evidence for inducible recruitment of Wiskott-Aldrich syndrome protein to T cell receptor-CD3 complex in Jurkat T cells

Pussadee Paensuwan,[1] Jatuporn Ngoenkam,[1] Boonruang Khamsri,[1] Kanlaya Preechanukul,[1] Donruedee Sanguansermsri[1] and Sutatip Pongcharoen[2,3]

Summary

Background: **The engagement of the T cell receptor (TCR)-CD3 complex induces the formation of multiple signalling complexes, which are required for actin cytoskeletal rearrangement. The Wiskott-Aldrich syndrome protein (WASp) is a key regulator of actin polymerization that is recruited to the TCR activation site. Since WASp is a binding partner of adaptor protein Nck, which is recruited directly to the TCR CD3ε subunit upon TCR ligation, therefore we proposed that the direct recruitment of Nck to TCR-CD3 may also bring WASp directly to TCR-CD3.**

Objective: **The aim of this present study was to assess the distribution of WASp, in relation to Nck, to the TCR-CD3ε complex.**

Methods: **Jurkat T cells were stimulated with anti-TCR antibody and then the cell lysates were immunoprecipitated with anti-CD3 antibody before immunoblotting with antibodies specific to WASp, Nck1, Nck2, SLP-76 and CD3ε molecules.**

Results: **WASp was recruited to SLP-76 and also directly to the TCR-CD3 complex upon TCR triggering. The inducible recruitment of WASp to the TCR-CD3 complex is partially dependent of tyrosine phosphorylation.**

Conclusions: **The present findings provide an alternative mechanism of WASp recruitment to the site of TCR activation that may be involved in recruitment of Nck.** ***(Asian Pac J Allergy Immunol 2015;33:189-95)***

Keywords: *WASp, TCR, CD3, conformational change, T cell activation*

From 1. Department of Microbiology and Parasitology, Faculty of Medical Science;
2. Department of Medicine, Faculty of Medicine
3. Center of Excellence in Medical Biotechnology, Faculty of Medical Science; Naresuan University, Pitsanulok 65000 Thailand
Corresponding author: Sutatip Pongcharoen
E-mail: sutatipp@nu.ac.th
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Introduction

The engagement of the T-cell antigen receptor (TCR) with its cognate antigenic peptide presented on a major histocompatibility complex (MHC) of antigen presenting cells (APC) results in the formation of signalling molecules critical for initiating the multiple signal transduction pathways and also for organizing the actin cytoskeletal rearrangements. The latter event is important for cellular shape change, movement, immunological synapse formation, and eventually for T cell activation.[1,2] The cytoskeletal rearrangement, in particular, filamentous actin (F-actin) is enriched at the interface of the conjugation between T cells and APC. Interference of actin reorganization by using actin polymerization inhibitors can result in impaired T cell functions.[3-5]

Various molecules are involved in connecting signals from TCR to actin rearrangement.[6-10] Following TCR engagement, protein tyrosine kinases are activated, which are required to phosphorylate many downstream substrates, including TCR-CD3 components (TCRζ, CD3ε, CD3δ and CD3γ) and multiple effector molecules.[5] Among other effector proteins, the transmembrane adaptors LAT (linker for activation of T cells) and SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa) are phosphorylated. Once phosphorylated, these two adaptor proteins recruit various kinase enzymes and adaptor proteins to form multi-protein signalling complexes.[11,12] Of these, adaptor protein Nck (non-catalytic region of tyrosine kinase) and its associated molecule WASp (Wiskott-Aldrich syndrome

protein) are recruited to the phosphorylated SLP-76 in the vicinity of the activated TCR.[5,13] Here, WASp is activated by the Rho family GTPase Cdc42 and subsequently leads to initiation of actin filament formation.[14]

Nck is a cytosolic adaptor protein that couples signals from the transmembrane receptor to downstream effectors for regulating the actin cytoskeletal organization.[15,16] Two members of the Nck family, Nck1/Nckα and Nck2/Nckβ (also known as Grb4), are identified in humans.[17] Previously, we have defined the non-overlapping functions of these proteins in activation of human T cells in which Nck1 plays a more dominant role than Nck2.[18, 19] Nck contains three consecutive Src homology 3 (SH3) domains at N-terminus followed by a SH2 domain at the C-terminus.[20] The SH2 domain of Nck binds to phosphotyrosine-containing proteins, whereas the SH3 domains bind to proline-rich sequences (PRS) within target proteins.[17, 21] In T cells, the SH2 domain of Nck is recruited to the TCR-proximal signalling complex by interacting with SLP-76[13] while the C-terminal of the SH3 domains of Nck binds to WASp for controlling actin polymerization.[22] Indeed, Nck is recruited not only to SLP-76 but also directly to the TCR CD3ε subunit upon TCR ligation.[23] Accordingly, it has been proposed that the direct recruitment of Nck to TCR-CD3 may also bring a signalling effector involved in the actin cytoskeletal reorganization directly to TCR-CD3.[23] Therefore, the aim of this present study was to assess the distribution of WASp, a key regulator of the actin filament formation, in the TCR-CD3ε. Here, we demonstrated that there are two distinct pathways in recruitment of WASp to the TCR activation site: the first is to SLP-76 and the second is directly to TCR-CD3ε.

Methods

Antibodies

Mouse anti-CD3 (OKT-3) antibody was purchased from e-Bioscience (eBioscience, San Diego, CA, USA). Mouse anti-TCR (clone C305) monoclonal antibody (mAb) was supplied from Upstate (Upstate Biotechnology, Lake Placid, NY, USA). Anti-Nck1 and anti-SLP-76 antibodies were from Cell Signalling (Cell Signalling Technology, Danver, MA, USA). Anti-Nck2 antibody was purchased from Abnova (Abnova Corp., Taipei, Taiwan). Anti-WASp antibody was supplied from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunoprecipitation and western blotting

A total of $3x10^7$ cells were washed with PBS. Cells were on starvation for 1 hr in 0.5 ml serum-free medium. Cells were left unstimulated or stimulated with anti-TCR antibody (C305) for the indicated time. The optimal concentration of C305 antibody for stimulation was determined by titration. After stimulation, cell pellets were lysed in ice-cold lysis buffer (20 mM TrisHCl (pH 8), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 500 μM sodium orthovanadate, 1 mM NaF, and 0.5% Brij 96) for 15 min on ice. After centrifugation, the lysates were incubated with 1 μg of desired antibody for 2 h at 4°C, rotating. Then, protein G-Sepharose beads (GE Healthcare, Uppsala, Sweden) were added and further incubated overnight at 4 °C, rotating. Immunoprecipitates were washed three times with lysis buffer. At the last washing step, the supernatant was discarded by pipetting and left only about 40 μl of lysis buffer in the tube. Ten microlitters of 4X non-reducing buffer were added and the samples were boiled at 95°C for 5 min to elute proteins from beads.

Western blotting

After the immunoprecipitation process, the eluted proteins and lysates were resolved on a 10% acrylamide gel and then transferred onto a PVDF membrane according to standard protocol. The membrane was blocked with 10 ml of RapidBlock solution (Amresco, Solon, Ohio, USA) for 10 min. The membrane was then incubated with primary monoclonal antibody and followed by secondary antibody. The membrane was developed using enhanced chemiluminescence reagent (ECL: Bio-Rad, USA) and was then analyzed by ImageQuant LAS 4000 imaging system (GE healthcare).

Results

WASp is recruited to TCR upon TCR-CD3 conformational change

In many cell types, WASp is associated with Nck, which is essential for controlling actin polymerization.[14,24,25] In this present study, we examined the binding of both Nck proteins with WASp by immunoprecipitation with anti-WASp antibody and immoblotting with anti-Nck1 and -Nck2 antibodies. As shown in Figure 1A, co-precipitation of both Nck proteins with WASp was found in unstimulated cells and did not increase

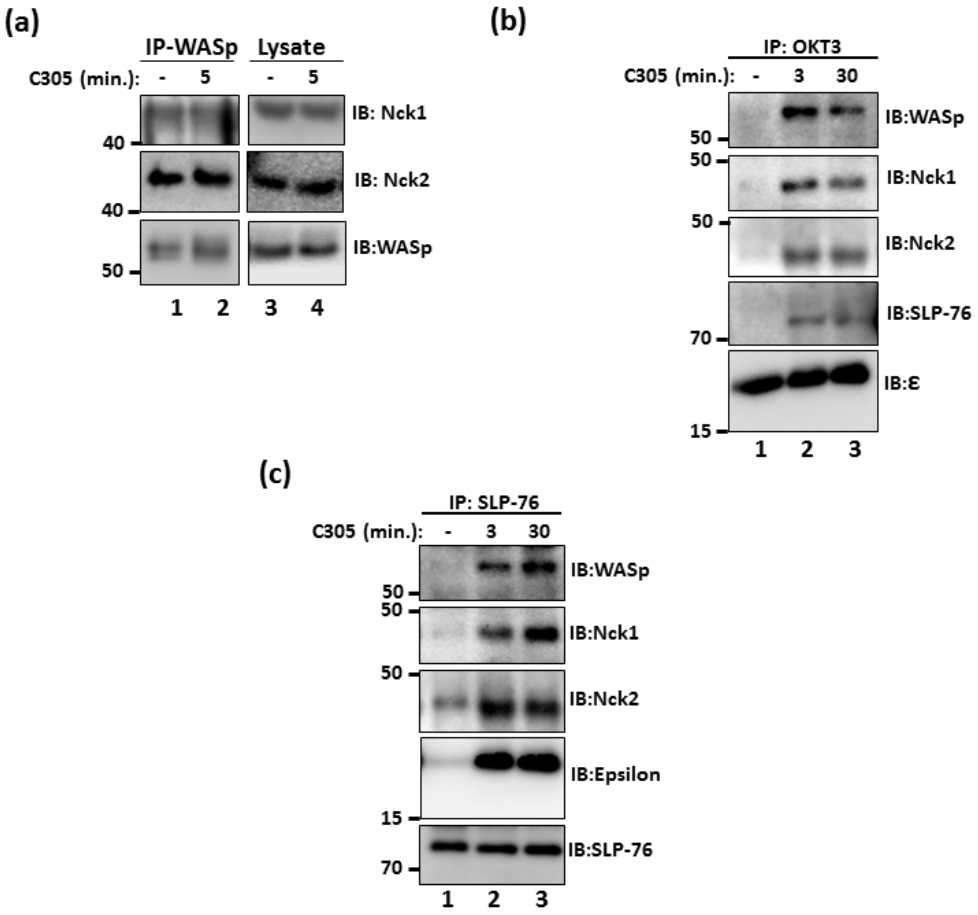

Figure 1. Engagement of TCR resulted in the recruitment of WASp to TCR-CD3ε.
A) Nck1 and Nck2 are constitutively associated with WASp. Jurkat cells were unstimulated or stimulated for 5 min with 1 μg/ml anti-TCR antibody (C305). Immunoprecipitation (IP) was done with anti-WASp antibody followed by immunoblotted (IB) with corresponding antibodies. The membrane was reprobed with anti-WASp antibody as a loading control. The data are representative of two independent experiments.
B) WASp is directly recruited to the TCR-CD3 complex upon stimulation. Jurkat T cells were unstimulated (-) or stimulated for 3 and 30 min with 1 μg/ml anti-TCR antibody (C305). Total cell lysate was immunoprecipitated with anti-CD3ε antibody and immunoblotted with corresponding antibodies. The membrane was reprobed with anti-CD3ε antibody as a loading control. The data are representative of three independent experiments.
C) Antibody engagement of TCR-CD3 induces the association of Nck with SLP-76. Jurkat T cells were treated as in (B). The total cell lysates were precipitated with anti-SLP-76 antibody and blotted with corresponding antibodies. Anti-SLP-76 antibody served as a loading control. The data are representative of two independent experiments.

after T cell stimulation. This indicated a constitutive interaction between these Nck proteins with WASp.

It has been found that engagement of TCR by its cognate pMHC ligands and antibodies against TCR and CD3 led to the conformational change of the CD3ε subunit.[23] This event is revealed by the exposure of PRS at the cytoplasmic tail of CD3ε, which is available for recruitment of Nck via its N-terminal SH3 (SH3.1) domain to the TCR-CD3 complex. In accordance with previous findings,[23] both adaptor proteins Nck1 and Nck2 were recruited to CD3ε-PRS upon TCR triggering (Figure 1B, second and third panels).

Since Nck was directly recruited to CD3ε upon TCR triggering, and it was also associated with WASp, we postulated that the recruitment of Nck to CD3ε may be accompanied by the recruitment of WASp to the TCR-CD3 complex. To clarify this issue, TCR-CD3 complexes of the cell lysates from unstimulated and stimulated cells were

immunoprecipitated with the anti-CD3 antibody (OKT3) and immunoblotted with antibody against WASp. As expected, we found the co-precipitation of WASp with TCR-CD3 upon T cell activation (Figure 1B, first panel). This suggests that the TCR engagement induced the association of Nck with CD3 and simultaneously mediated the recruitment of WASp to TCR-CD3 complexes. Interestingly, we also observed the recruitment of SLP-76 to CD3ε after TCR induction (Figure 1B, forth panel).

After T cell stimulation, the phosphorylated SLP-76 recruits many proteins, such as Nck and WASp. Thus, the distribution of WASp in SLP-76 was also monitored. We precipitated SLP-76 proteins from the cell lysates of unstimulated and stimulated cells followed by immunoblotting with antibody against WASp. As shown in Figure 1C, WASp as well as Nck1 and Nck2 were recruited to SLP-76 following TCR engagement. Furthermore, we also found the co-precipitation of CD3ε with SLP-76 in stimulated T cells (Figure 1C, forth panel). This confirmed that SLP-76 was recruited to the TCR-CD3 complex after TCR triggering. Taken together, we suggest that there are two pathways for recruitment of WASp to TCR activation sites: first, WASp is recruited to SLP-76, and second, WASp is directly recruited to TCR-CD3 complexes. In these pathways, Nck might act as a bridge to recruit WASp to the TCR activation site.

The recruitment of WASp to the TCR-CD3 complex is partially dependent on CD3ε tyrosine phosphorylation

The early event after TCR engagement is the phosphorylation of ITAMs within the TCR-CD3 complex mediated by src family protein tyrosine kinase (PTK) members Lck and Fyn. This is an essential mechanism to initiate the signal transduction from TCR to downstream signalling pathways. In a previous report, CD3 conformational change as revealed by the binding of Nck to the TCR-CD3 complex was found to occur before and independent of tyrosine phosphorylation at ITAMs of the TCR-CD3 complex.[23] Thus, we hypothesized that the association of WASp with TCR is similar to Nck. We tested this hypothesis by using a phosphatase inhibitor (pervanadate, PV) and protein tyrosine kinase (PTK) inhibitor (PP2). PV enhanced the level of tyrosine phosphorylation independent of TCR triggering[26] while PP2 inhibited the src family, such as Lck and Fyn.[27]

Jurkat cells stimulated with anti-TCR antibody caused an increase of CD3ε tyrosine phosphorylation and substantially increased in response to PV treatment (Figure 2, second panel). In contrast to anti-TCR antibody (C305), Jurkat cells stimulated with PV alone did not promote the conformational change of CD3,[23] which prevent the association of WASp with TCR-CD3 (Figure 2, first panel). However, CD3ε tyrosine phosphorylation was markedly inhibited in cells stimulated with anti-TCR antibody in the presence of PP2 (Figure 2, second panel). A previous study has demonstrated a dispensable role of tyrosine phosphorylation on the conformational change of TCR-CD3 complex. [23] Interestingly, we found less WASp binding to TCR-CD3 in the absence of tyrosine phosphorylation (Figure 2, first panel). Altogether, binding of WASp to the TCR-CD3 complex was due to CD3 conformational change and its recruitment to TCR would depend on PTK activity.

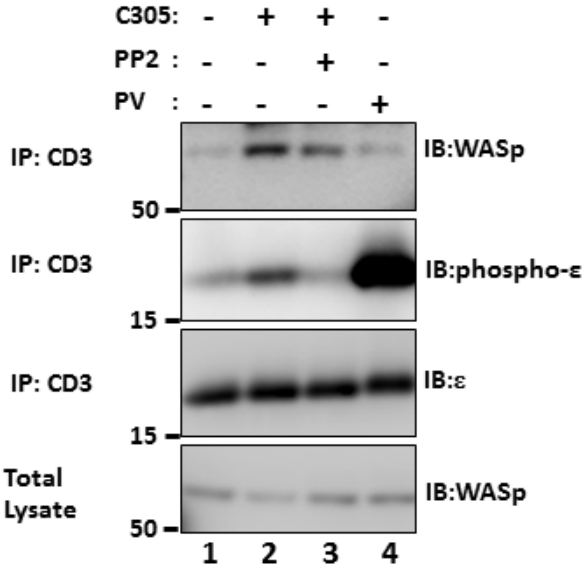

Figure 2. Inhibition of tyrosine phosphorylation at CD3ε subunit partially impaired the recruitment of WASp to the TCR-CD3 complex. CD3ε immunoprecipitation was performed from nonstimulated (0 min), C305-stimulated (5 min), C305-stimulated (5 min) in the presence of PP2, or pervanadate-stimulated (PV) Jurkat cells. IB was performed with anti-WASp or anti-phospho ε antibody. The membrane was reprobed with anti-CD3ε antibody as a loading control. A fraction of the total lysate was run in parallel. The data are representative of three independent experiments.

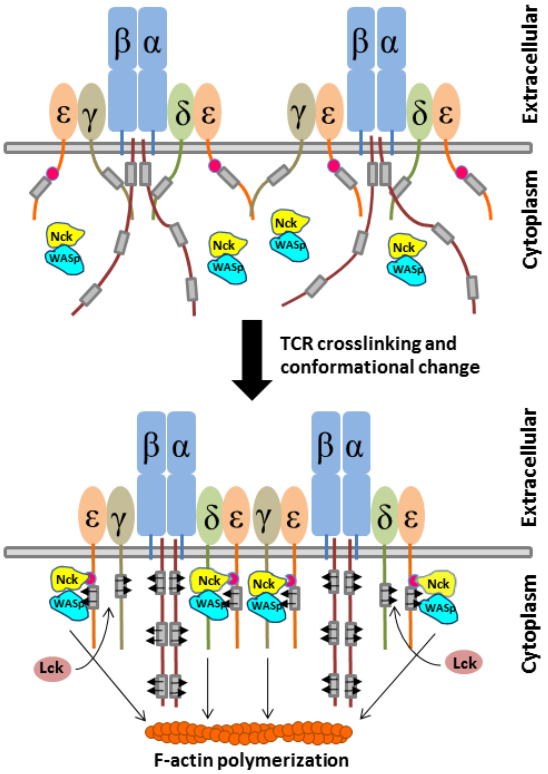

Figure 3. Schematic diagram of WASp recruitment to TCR-CD3 complex upon T cell activation. The activation of TCR-CD3 complex results in both clustering and a conformational change of the TCR-CD3 molecules. TCR-CD3 clustering could mediate the activation of src kinases Lck, which then phosphorylate the ITAM (indicated as pinned boxes). A conformational change of cytoplasmic tails of CD3ε subunits leads to the exposure of proline-rich sequence (PRS) (indicated as red dots). Subsequently, Nck in association with Wiskott-Aldrich syndrome protein (WASp) is directly recruited to the exposed PRS at CD3ε, which would be necessary for recruitment of WASp-interacting protein (WIP) and CDC42 (did not show), thereby initiating F-actin polymerization.

Discussion

TCR-induced cytoskeleton reorganization is essential for T cell responses, such as cellular shape change, movement, proliferation, and activation. The key molecules, including SLP-76, Nck, WASp, WAVE2, and ADAP, coordinate and function in the actin polymerization process.[8,10,28,29] At the site of the T cell/APC interface, these proteins are dynamically accumulated at the periphery of the T cells and colocalize with the polymerized actin in the vicinity of the activated TCR.[8] Among these proteins, WASp is a key regulator of the actin cytoskeletal remodelling. Typically, the regulation F-actin formation by WASp relies on two processes: the recruitment of WASp to the SLP-76 signalling complex and the activation of WASp by the Rho family GTPase Cdc42.[30] Thus, it has been suggested that the recruitment of WASp to the plasma membranes is mediated by the SLP-76 protein.[14] In addition, molecular imaging analysis also reveals the co-localization of WASp with TCR-associated protein tyrosine kinase ZAP-70 (ζ-chain-associated protein of 70 kDa) after T cell activation, indicating that WASp is recruited to the TCR activation site upon activation.[8]

Our present study provided more direct biochemical evidence that WASp was directly recruited to the TCR-CD3 complex upon TCR engagement as proposed before.[23] Moreover, the distribution of WASp in the SLP-76 signalling complex upon activation was also confirmed in this present study. Since WASp and Nck were co-precipitated with CD3 and SLP76 molecules, the recruitment of WASp to TCR-CD3 and SLP-76 might be mediated by Nck. The N-terminal SH3 domain of Nck is essential for interaction with PRS of CD3ε,[23] and the Nck SH2 domain is crucial for association with SLP-76.[31] Additionally, we found that WASp was constitutively associated with Nck as suggested previously.[22] WASp contains the proline-rich region, which can interact with the C-terminus SH3 domain of Nck.[17]

Tyrosine phosphorylation of ITAMs by Src family PTKs elicited upon TCR triggering is the earliest event and essential for TCR-mediated signal transductions.[32] Interestingly, the binding of Nck to PRS of CD3ε as a result of CD3 conformational change is earlier and is independent of tyrosine phosphorylation.[23] However, imaging analysis revealed that the recruitment of Nck to CD3 was abrogated after inhibition of the Src kinase activation,[8] which contradicted previous results.[23] Thus, such binding seems to depend on TCR phosphorylation. In the present study, we have investigated the requirement of tyrosine phosphorylation on the binding of WASp and CD3. Src kinase inhibitor PP2 was used to inhibit proximal tyrosine phosphorylation. In this present study, we found that there was less WASp binding to TCR-CD3 complex when CD3ε phosphorylation was blocked. Furthermore, when using the phosphatase inhibitor PV to enhance CD3ε tyrosine phosphorylation without inducing the change of the TCR-CD3 complex,[23] we detected no recruitment of WASp to TCR-CD3. Thus, our results suggested

that the recruitment of WASp to TCR required both conformational change and, to a lesser extent, tyrosine phosphorylation of CD3ε subunits. A schematic diagram of WASp recruitment to TCR is shown in Figure 3.

In summary, using a biochemical approach, we provided an alternative pathway of WASp recruitment to the plasma membranes in close proximity to the activated TCR. In addition to SLP-76, WASp was directly recruited to the TCR-CD3 complex upon TCR engagement and partially dependent of tyrosine phosphorylation. We suggest that the recruitment of WASp to these different sites was mediated by Nck molecules.

Conflict of interest

The authors have no conflicting interests.

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