

# Mechanism of Recruitment of WASP to the Immunological Synapse and of Its Activation Following TCR Ligation

Yoji Sasahara,<sup>1,4</sup> Rima Rachid,<sup>1,4</sup> Michael J. Byrne,<sup>1</sup> Miguel A. de la Fuente,<sup>1</sup> Robert T. Abraham,<sup>2</sup> Narayanaswamy Ramesh,<sup>1,3</sup> and Raif S. Geha<sup>1,3</sup>

<sup>1</sup>Division of Immunology  
Children's Hospital and  
Department of Pediatrics  
Harvard Medical School  
Boston, Massachusetts 02115

<sup>2</sup>Department of Pharmacology  
Duke University Medical Center  
Durham, North Carolina 27710

## Summary

**F-actin polymerization following engagement of the T cell receptor (TCR) is dependent on WASP and is critical for T cell activation. The link between TCR and WASP is not fully understood. In resting cells, WASP exists in a complex with WIP, which inhibits its activation by Cdc42. We show that the adaptor protein CrkL binds directly to WIP. Further, TCR ligation results in the formation of a ZAP-70-CrkL-WIP-WASP complex, which is recruited to lipid rafts and the immunological synapse. TCR engagement also causes PKC $\theta$ -dependent phosphorylation of WIP, causing the disengagement of WASP from the WIP-WASP complex, thereby releasing it from WIP inhibition. These results suggest that the ZAP-70-CrkL-WIP pathway and PKC $\theta$  link TCR to WASP activation.**

## Introduction

Interaction between the T cell receptor (TCR) and peptide-loaded major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs) induces the formation of molecular clusters at the contact site that are enriched in filamentous actin (F-actin) (Grakoui et al., 1999; Penninger and Crabtree, 1999). These supramolecular activation clusters (SMACs), also named immunological synapses (IS), contain several signaling components. They include src and Syk family kinases, PKC $\theta$  and Cdc42-GTP (Bromley et al., 2001). The IS also contains adaptor proteins such as SLP-76, Fyb, and Nck that are linked directly or indirectly to proteins such as WASP, Ena/VASP family members that are involved in actin polymerization (Krause et al., 2000; Monks et al., 1998). The accumulation of F-actin at the T cell-APC interface is thought to stabilize a continuous contact between T cells and APCs, which is required for optimal T cell activation. Inhibition of actin polymerization by cytochalasin blocks formation of the immunological synapse and T cell activation (Wulfiging and Davis, 1998).

WASP, the product of the gene mutated in Wiskott-

Aldrich syndrome (WAS), is expressed only in hematopoietic cells and is the first identified member of an expanding family of proteins involved in signaling and cytoskeletal organization that includes N-WASP and Scar/WAVE (Derry et al., 1994; Machesky and Insall, 1998; Miki et al., 1996). WASP has an N-terminal Ena/VASP homology domain 1 (EVH1) domain, a Cdc42/Rac GTPase binding domain (GBD), a proline-rich domain, a G-actin binding verprolin homology (VH) domain, a cofilin homology (CH) domain, and a C-terminal acidic (A) segment. WASP interacts with WIP via its N-terminal EVH1 domain (Ramesh et al., 1997), with Cdc42-GTP via its GBD domain, with multiple SH3 domain-containing proteins that include Nck via its proline-rich region, and with actin and the Arp2/3 complex via its VCA domain (Abo, 1998). WASP exists in cells in a closed inactive conformation due to intramolecular interactions that involve the C-terminal acidic domain and a basic region that precedes the GBD domain. Binding of Cdc42-GTP is thought to cause a conformational change in WASP, which allows the VCA domain to interact with and activate the Arp2/3 complex (Higgs and Pollard, 2000; Kim et al., 2000; Rohatgi et al., 2000). WASP plays a critical role in T cell activation and actin reorganization. T cells from WAS patients and WASP<sup>-/-</sup> mice are severely deficient in their ability to increase their F-actin content, secrete IL-2, and proliferate following TCR ligation (Gallego et al., 1997; Snapper et al., 1998; Zhang et al., 1999).

WIP is a 503 aa long proline-rich protein expressed at high levels in lymphoid tissues (Ramesh et al., 1997). WIP binds actin via its VH domain (aa 1–151) and WASP via its carboxy-terminal end (aa 416–488). A WIP-WASP/N-WASP complex is readily detected in resting cells (Martinez-Quiles et al., 2001). In lymphocytes, >95% of WASP is complexed with WIP (our unpublished data). WIP inhibits Cdc42-mediated activation of N-WASP, suggesting that one function of WIP is to stabilize WASP/N-WASP in their inactive closed conformation (Martinez-Quiles et al., 2001). Another function of WIP is to stabilize actin filaments (Martinez-Quiles et al., 2001). WIP, like WASP, plays an important role in T cell activation. T cells from WIP<sup>-/-</sup> mice fail to proliferate, secrete IL-2, or increase their F-actin content after TCR ligation. Furthermore, WIP<sup>-/-</sup> T cells are deficient in conjugate formation with superantigen-presenting B cells and anti-CD3/ICAM-1-containing lipid bilayers and have a disorganized actin cytoskeleton (Anton et al., 2002).

Recent data shows that WASP localizes with F-actin to the IS where it is thought to be activated by Cdc42-GTP generated following activation of the exchange factor Vav and its recruitment to lipid membranes (Arudchandran et al., 2000). The mechanism(s) by which WASP is recruited to the IS and is released from WIP inhibition to initiate actin polymerization is not well understood. We show here that WIP binds to the adaptor protein CrkL and that following TCR ligation, a CrkL-WIP-WASP complex is recruited by ZAP-70 to lipid rafts and the IS. TCR ligation also causes PKC $\theta$ -dependent phosphorylation of WIP and disengagement of WASP

<sup>3</sup> Correspondence: narayanaswamy.ramesh@tch.harvard.edu (N.R.), raif.geha@tch.harvard.edu (R.S.G.)

<sup>4</sup> These authors contributed equally to this work.

from the WIP-WASP complex, releasing it from WIP inhibition.

## Results

### The Adaptor Protein CrkL Binds to WIP and Associates with ZAP-70 after TCR Ligation

In a search for potential links between the activated TCR-ZAP-70 complex and the WIP-WASP complex, we considered the possibility that an SH2-SH3 domain-containing adaptor protein may link phosphorylated ZAP-70 to the proline-rich WIP and WASP. Potential candidates included the CT10 regulator of kinase (Crk) family of adaptor proteins. The prototype, CrkII, has one N-terminal SH2 domain and two SH3 domains. The second SH3 domain (SH3.2) is alternatively spliced out to give rise to CrkI. A closely related member, CrkL (Crk like), resembles CrkII in that it also has two SH3 domains and is expressed at high levels in hematopoietic cells (Feller et al., 1998). GST-CrkII and GST-CrkL have been reported to associate via their SH2 domains with phosphorylated ZAP-70 (Gelkop and Isakov, 1999). WIP, but not WASP, contains two copies of the consensus motif P<sub>x</sub>LP<sub>x</sub>K/R, which binds to the N-terminal SH3 domain (SH3.1) of Crk proteins.

To determine if WIP and WASP bind to Crk proteins, we expressed CrkII and CrkL as GST fusion proteins and examined their ability to pull down Xpress-tagged recombinant WIP and *in vitro* transcribed and translated WASP. CrkI was not examined because it is an alternative splicing truncated product of CrkII. WIP bound to CrkL, but not to CrkII. (Figure 1A). This indicates direct interaction between WIP and CrkL. WASP failed to bind to either CrkL or CrkII (Figure 1A), even in the presence of the constitutively active Cdc42 mutant Cdc42L61 (data not shown). Using the yeast two-hybrid system, we found that the SH3.1 domain of CrkL binds to a region of WIP spanned by aa 321–415 (Figure 1B). This region contains the two consensus binding motifs for the Crk SH3.1 domain (aa 332–337 and 399–404) and is distinct from the WASP binding site (aa 416–488).

We next determined if CrkL associates with ZAP-70 and WIP in T cells. Western blotting of CrkL immunoprecipitates from Jurkat cells revealed that CrkL associated with ZAP-70 following anti-CD3 stimulation (Figure 1C). WIP coprecipitated with CrkL in unstimulated Jurkat T cells, and this association increased slightly after TCR ligation (Figure 1C). Similar results were obtained in PHA-derived T cell blasts derived from normal subjects (see Supplemental Figure S1 at <http://www.moleculer.org/cgi/content/full/10/6/1269/DC1>). WIP coprecipitated with CrkL from peripheral blood T cell blasts of a WAS patient with a point mutation in the WASP gene (C73Y), who does not express WASP protein (Figure 1D). These results suggest that WIP associates with ZAP-70 after TCR ligation and associates constitutively with CrkL independently of WASP.

### WIP and WASP Are Recruited to ZAP-70 after TCR Ligation

Since WIP exists as a preformed complex with WASP and since the WIP binding sites for CrkL and WASP are distinct, we examined the possibility that CrkL may

recruit the WIP-WASP complex to ZAP-70 following TCR ligation. We first probed ZAP-70 immunoprecipitates from Jurkat cells before and 3 min after anti-CD3 stimulation for CrkL, WIP, and WASP. As expected, anti-CD3 stimulation resulted in vigorous tyrosine phosphorylation of ZAP-70 (Figure 2A). CrkL and WIP became readily detectable in ZAP-70 immunoprecipitates after anti-CD3 stimulation. The weak association of these proteins with ZAP-70 in unstimulated cells may be explained by the presence of small amounts of phosphorylated ZAP-70 in these cells. We were unable to detect WASP in ZAP-70 immunoprecipitates.

In a further attempt to demonstrate association of ZAP-70 with WASP, we probed WASP immunoprecipitates for ZAP-70. Figure 2B shows that ZAP-70 coprecipitated weakly with WASP in unstimulated cells. The association of WASP and ZAP-70 increased after anti-CD3 stimulation, suggesting that WASP is recruited to ZAP-70 following TCR ligation. Probing of WASP immunoprecipitates for CrkL and WIP revealed that CrkL, like WIP, coprecipitated with WASP in unstimulated cells, suggesting that CrkL, WIP, and WASP exist as preformed complex, with WIP bridging WASP to CrkL. CrkI and CrkII were not detected in WASP immunoprecipitates (data not shown). Anti-CD3 stimulation resulted in decreased association of WASP with WIP and CrkL, suggesting that TCR ligation may perturb the WIP-WASP complex. Similar results were obtained in normal T cell blasts (see Supplemental Figure S1 at <http://www.moleculer.org/cgi/content/full/10/6/1269/DC1>). Taken together, our results suggest that a CrkL-WIP-WASP complex is recruited to phosphorylated ZAP-70 following TCR ligation.

### CrkL, WIP, and WASP Translocate to GEMs after TCR Ligation

Glycosphingolipids and cholesterol self-associate in plasma membrane microdomains known as glycolipid enriched microdomains (GEMs) or lipid rafts. GEMs localize at the IS in antigen-stimulated T cells (Bi et al., 2001) and are enriched in a number of molecules relevant for receptor signaling that leads to F-actin polymerization and IL-2 gene expression. Signaling molecules recruited to lipid rafts after TCR ligation include phosphorylated CD3 $\zeta$  and its associated ZAP-70. Since ZAP-70 associates with CrkL, WIP, and WASP in activated T cells, we examined whether these proteins translocate to GEMs following TCR ligation.

We used sucrose density gradient fractionation of Triton X-100 lysates from Jurkat cells to analyze the translocation of CrkL, WIP, and WASP to GEMs, which sediment in fractions 2–5 of the gradient, as evidenced by the presence of the glycosphingolipid GM1 in these fractions. Small amounts of 21 kDa phosphorylated CD3 $\zeta$ , ZAP-70, CrkL, WIP, and WASP were present in GEMs before stimulation. As expected, phosphorylated CD3 $\zeta$  and ZAP-70 translocated to GEMs after anti-CD3 stimulation. TCR ligation caused translocation of CrkL, WIP, and WASP to GEMs (Figure 3C).

### Translocation of WIP and WASP to GEMs Is Dependent on ZAP-70 and CrkL

We used ZAP-70-deficient P116 Jurkat cells to examine the role of ZAP-70 in the translocation of CrkL, WIP, and

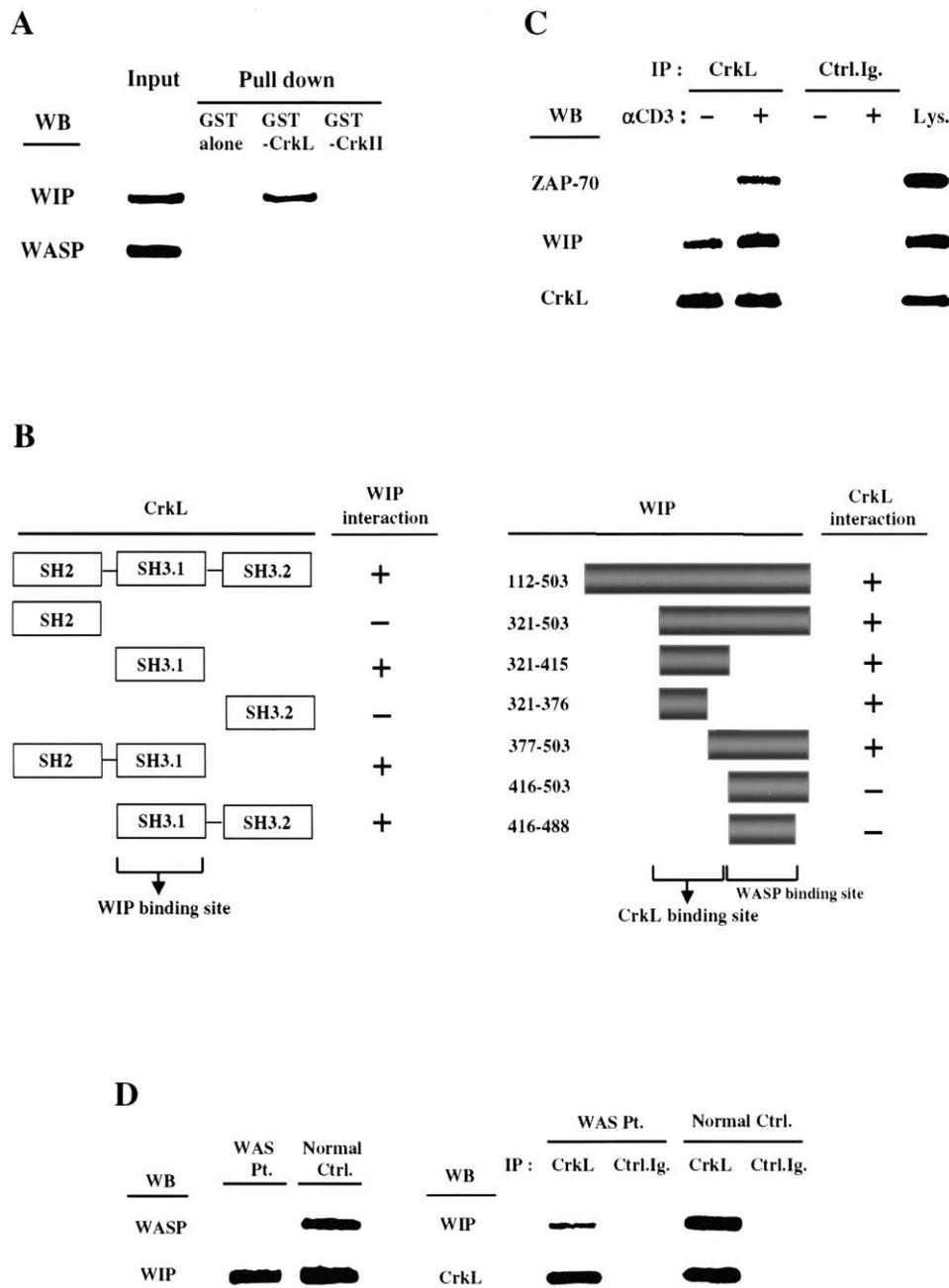


Figure 1. Interaction between CrkL and WIP

(A) Pull-down assay using GST-CrkL, GST-CrkII fusion proteins and GST with purified Xpress-tagged WIP or in vitro translated WASP. Bound proteins were probed with anti-Xpress or anti-WASP mAbs. The left lane in each panel was loaded with WIP or WASP protein ("Input"). (B) Mapping by yeast two-hybrid assay of the WIP binding site in CrkL (left panel) and of the CrkL binding site in WIP (right panel). (C) Coprecipitation of WIP and ZAP-70 with CrkL in unstimulated and anti-CD3-stimulated Jurkat cells. CrkL and control ("Ctrl. Ig.") immunoprecipitates were probed for ZAP-70, WIP, and CrkL. The right lane in each panel was loaded with cell lysates ("Lys."). (D) WIP association with CrkL is independent of WASP. Left: expression levels of WIP and WASP in lysates from T cells of a WAS patient ("pt.") with C73Y WASP mutation and a normal control ("Ctrl."). Right: CrkL and control immunoprecipitates from T cells of the patient and normal control were probed for WIP and CrkL.

WASP to GEMs. The parent P116 cell line was stably transfected with either Myc-tagged wt (wild-type) ZAP-70 or Myc-tagged kinase-dead (KD) ZAP-70. At least two clones that showed equivalent surface CD3 expression to wt Jurkat cells were studied for each construct, with similar results. Expression of ZAP-70 proteins in

the transfected P116 clones was confirmed by Western blotting (Figure 3A). In contrast to wild-type Jurkat cells, there was no translocation of CrkL, WIP, or WASP to GEMs in ZAP-70-deficient P116 cells (Figure 3B). Translocation of all three proteins to GEMs was restored in P116 cells reconstituted with wt ZAP-70, but not in P116

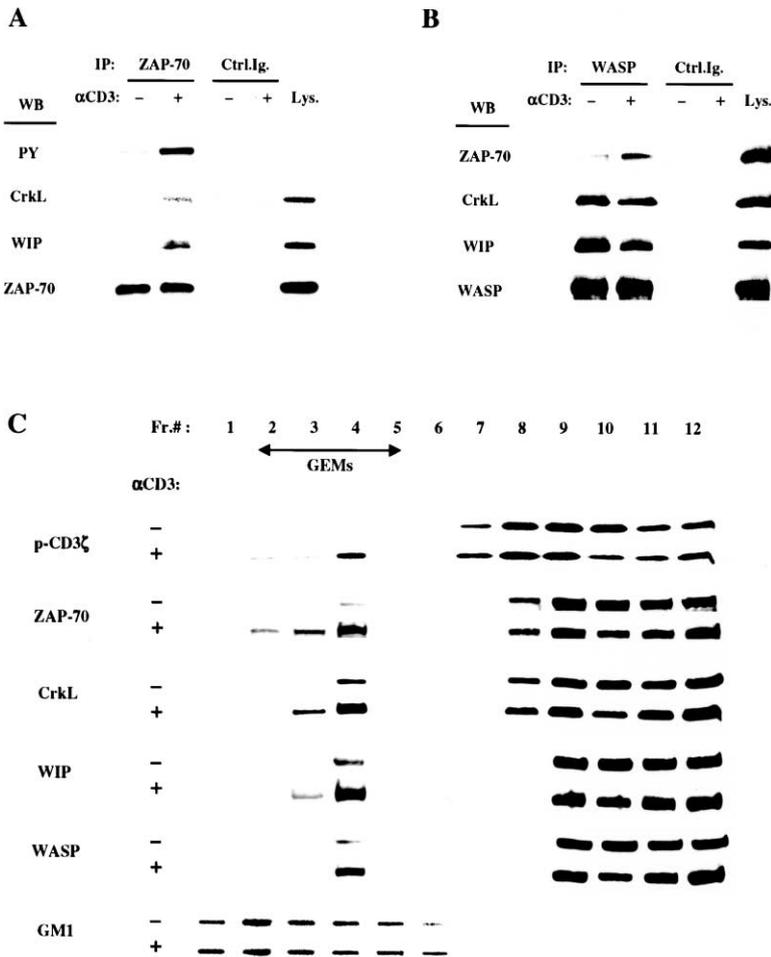


Figure 2. ZAP-70, CrkL, WIP, and WASP Form a Complex following TCR Ligation and Are Recruited to Lipid Rafts

(A) ZAP-70 immunoprecipitates from unstimulated and anti-CD3 stimulated Jurkat T cells and lysates ("Lys.") were probed with Abs to phosphotyrosine ("PY"), CrkL, WIP, and ZAP-70 as a loading control. (B) WASP immunoprecipitates and lysates from the same cells were probed for ZAP-70, CrkL, WIP, and WASP as a loading control. (C) Sucrose density gradient fractions of Triton X-100 lysates from unstimulated and anti-CD3 stimulated Jurkat cells were probed for CD3ζ, ZAP-70, CrkL, WIP, and WASP. The visualized 21 kDa band corresponds to phosphorylated CD3ζ. Cholera toxin B subunit was used to probe for GM1 that constitutively resides in GEMs as a loading control.

cells reconstituted with KD ZAP-70. These results suggest that ZAP-70 and its kinase activity are essential for the translocation of the CrkL-WIP-WASP complex to lipid rafts.

To examine the role of CrkL in the translocation of WIP and WASP to GEMs, Jurkat T cells were stably transfected with a CrkL deletion mutant that lacked the SH3.1 domain that is essential for WIP binding (CrkLΔSH3.1), or vector alone. The mutant would compete with native CrkL for ZAP-70 binding. Two clones that showed equivalent surface CD3 expression to wt Jurkat cells were studied with similar results. Western blotting revealed that the mutant was expressed 2.5-fold higher than endogenous protein (Figure 3C). Mutant CrkL, like native CrkL, translocated to GEMs after TCR ligation, consistent with the notion that the mutant can be recruited by ZAP-70 via its SH2 domain. In contrast, WIP and WASP translocated poorly to GEMs in CrkLΔSH3.1-transfected cells (Figure 3D).

**WIP and WASP Translocate to the T Cell-APC Interface in a ZAP-70- and CrkL-Dependent Manner**  
Since CrkL, WIP, and WASP translocate to GEMs, we examined whether they also localize to the T cell-APC interface. Jurkat T cells were incubated with Raji B cells in the presence of the superantigen SEE, and T:B cell conjugates were examined for localization of CrkL, WIP, WASP, and F-actin after 10 min. CrkL, WIP, and WASP

all translocated to the T cell-APC interface (Figure 4A and Table 1). As expected, F-actin also accumulated at the interface.

Translocation of CrkL, WIP and WASP to the T cell-APC interface was dependent on ZAP-70, because it was markedly diminished in ZAP-70-deficient P116 cells. Both the percentages of conjugates that contained these proteins at the interface and the amount of accumulated proteins were decreased (Figure 4A and Table 1). Conjugate formation by these cells was normal (data not shown). Consistent with previous data (Morgan et al., 2001), there was a much weaker accumulation of F-actin at the interface in P116 cells. Translocation of CrkL, WIP, and WASP and F-actin accumulation at the interface was completely corrected in P116 cells reconstituted with wt ZAP-70, but remained deficient in P116 cells reconstituted with KD ZAP-70. Translocation of WIP and WASP to the T cell-APC interface was also dependent on CrkL, because it was markedly diminished in CrkLΔSH3.1-transfected Jurkat cells (Figure 4A and Table 1). Conjugate formation by these cells was normal (data not shown).

**ZAP-70 and CrkL Play an Important Role in Cellular F-Actin Increase and IL-2 Synthesis following TCR Ligation by Superantigen Presented by APCs**  
We next investigated the role of ZAP-70 and CrkL in the increase of total cellular F-actin content and in IL-2

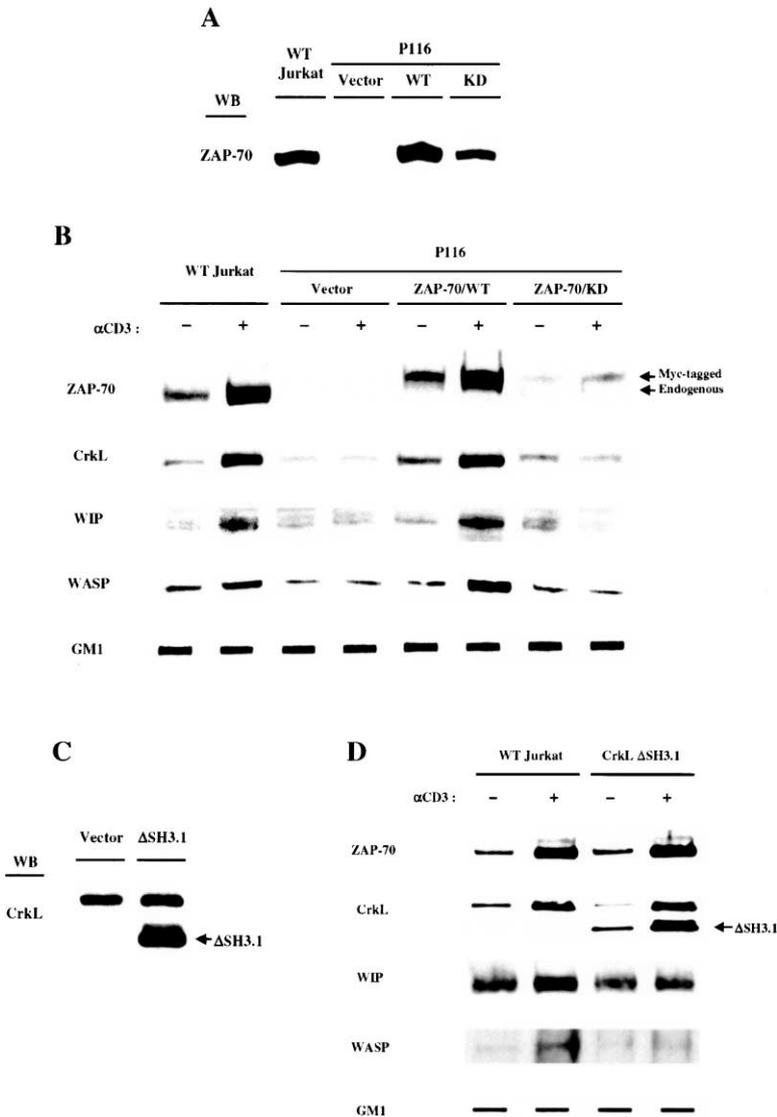


Figure 3. Recruitment of WIP-WASP Complex to Lipid Rafts Is Dependent on ZAP-70 and CrkL

(A) Expression of ZAP-70 protein in wt Jurkat cells and in P116 clones stably transfected with vector alone, wild-type (“WT”) ZAP-70, and a kinase-dead (“KD”) ZAP-70 mutant. Lysates were probed with anti-ZAP-70 mAb. (B) Recruitment of CrkL, WIP, and WASP to lipid rafts in Jurkat cells, P116 cells, and P116 reconstituted cells. Fractions 2–5 of the sucrose gradient were pooled and probed for ZAP-70, CrkL, WIP, and WASP and for GM1 as control. (C) Expression of CrkL protein in Jurkat clones stably transfected with vector alone or with a CrkL mutant lacking the SH3.1 domain (“ΔSH3.1”). Lysates were probed with anti-CrkL mAb. (D) Recruitment of ZAP-70, CrkL, WIP, and WASP to lipid rafts in CrkLΔSH3.1-transfected Jurkat cells and controls.

synthesis following TCR ligation. P116 cells failed to increase their F-actin content after anti-CD3 stimulation and to secrete IL-2 following stimulation with APCs and SEE. The latter result is consistent with results obtained with anti-CD3 stimulation (Williams et al., 1998). These deficits were completely corrected by reconstitution with wt ZAP-70, but not with KD ZAP-70 (Figures 4B and 4C). CrkLΔSH3.1-transfected Jurkat cells were also markedly impaired in their capacity to increase their F-actin content (Figure 4B and Supplemental Figure S2 at <http://www.molecule.org/cgi/content/full/10/6/1269/DC1>) and secrete IL-2 following TCR ligation (Figure 4C), but had normal calcium fluxes (data not shown).

#### WIP Is Phosphorylated by PKCθ following TCR Ligation

Translocation of the WIP-WASP complex to the IS brings it into proximity with Cdc42-GTP. However, since almost all of the WASP (>95%) in resting T cells is complexed with WIP, which inhibits its activation by Cdc42, we investigated potential mechanisms of WASP activation following TCR ligation. WIP, but not WASP, contains a

single consensus PKC phosphorylation motif (RxxS/TxR) that surrounds S488. PKCθ, the major PKC in T cells, is activated and translocates to GEMs and the IS following TCR ligation (Bi and Altman, 2001). This raised the possibility that PKCθ phosphorylation of WIP may perturb the WIP-WASP complex. In addition to the C45 antiserum which recognizes the carboxy-terminal 45 aa of WIP (aa 459–503), we have raised another antiserum, C14, against a 14 aa peptide of WIP, <sup>483</sup>ESRSGSNRRERGGAP<sup>496</sup>, which contains S488 (Figure 5A). C14 Ab reactivity was markedly decreased 5 min after treatment of Jurkat cells with anti-CD3 and was restored 30 min later (Figure 5B). In contrast, C45 Ab reactivity remained unchanged. Reactivity with C14 Ab was fully restored by treatment of lysates with alkaline phosphatase (Figure 5C). Similar results were obtained in peripheral blood T cells (data not shown). These findings indicate that WIP is phosphorylated after TCR ligation and that C14 Ab recognizes a nonphosphorylated WIP epitope, whereas C45 Ab is insensitive to phosphorylation.

To explore the identity of the enzyme that results in WIP phosphorylation, we first examined the effect of

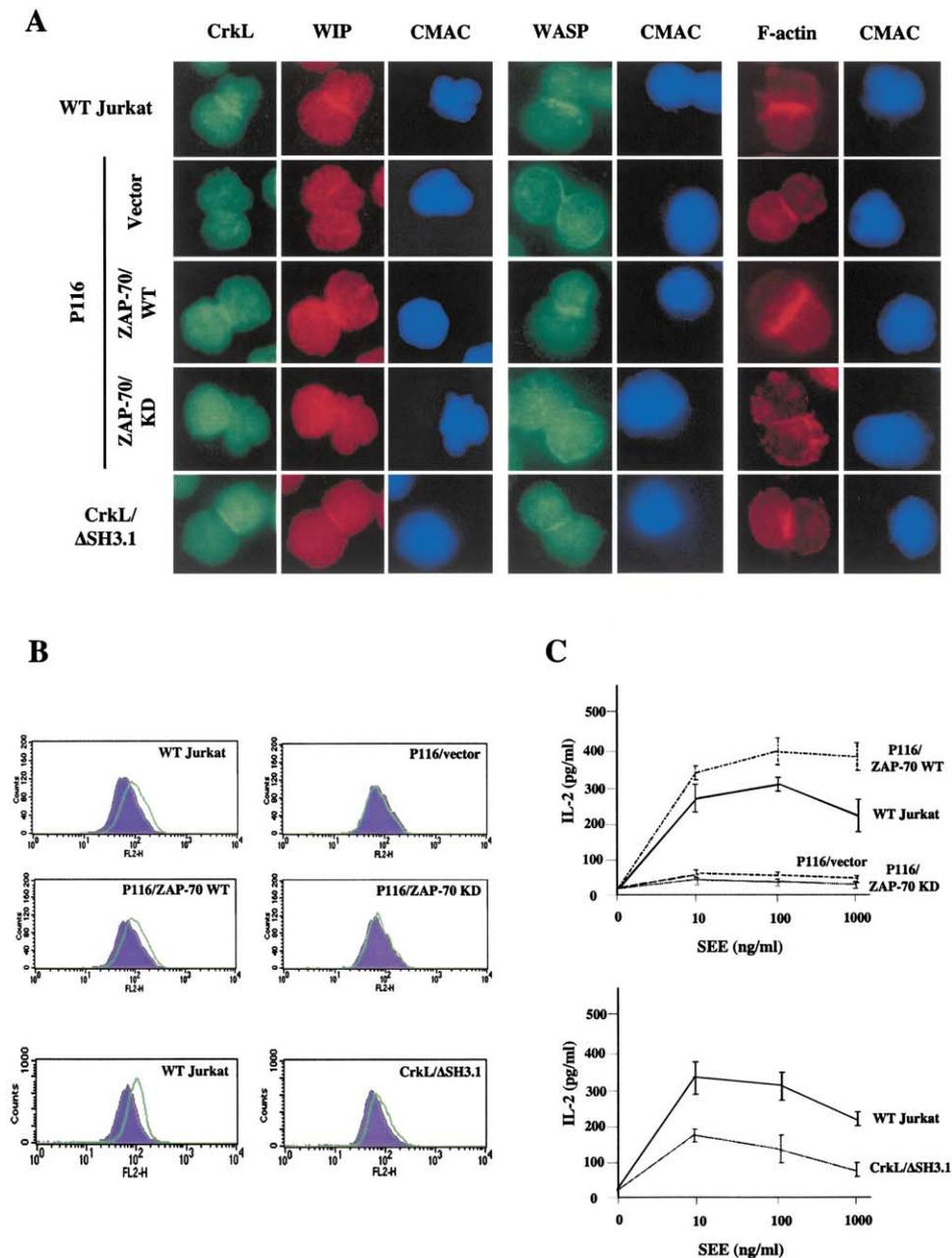


Figure 4. Recruitment of CrkL, WIP, WASP, and F-Actin at the T Cell-APC Interface, F-Actin Content, and IL-2 Secretion Are Dependent on ZAP-70 and CrkL

(A) Intracellular immunofluorescence staining for CrkL, WIP, WASP, and F-actin in T cells stimulated by SEE (5  $\mu$ g/ml), and Raji B cells preloaded with CMAC (blue). Cells were stained simultaneously for CrkL and WIP. In separate experiments, cells were stained for WASP and F-actin. Magnification is 600 $\times$ .

(B) FACS analysis of intracellular F-actin content of cells following CD3 crosslinking. Representative results of three independent experiments are shown.

(C) IL-2 secretion by T cells stimulated with SEE and Raji B cells. Supernatants collected at 24 hr were assayed for IL-2 by ELISA. Means  $\pm$  SD of three independent experiments are shown.

selective PKC inhibitors. Pretreatment of Jurkat cells with rottlerin, an inhibitor of the nonclassical PKCs  $\delta$  and  $\theta$  (Villalba et al., 1999), inhibited WIP phosphorylation following TCR ligation, as evidenced by retention of C14 Ab reactivity (Figure 5D). In contrast, G06976, an inhibitor of the classical calcium-dependent PKCs  $\alpha$ ,  $\beta$ , and  $\gamma$  (Martiny-Baron et al., 1993), and the PKA inhibitor 14-22 amide had no effect (Figure 5D and data not shown).

To ascertain the role of PKC $\theta$  in WIP phosphorylation, we examined splenic T cells from PKC $\theta^{-/-}$  mice. Anti-CD3 stimulation resulted in loss of C14 Ab reactivity in T cells from wt mice. In contrast, there was no loss of C14 Ab reactivity in T cells of PKC $\theta^{-/-}$  mice (Figure 5E). ZAP-70, SLP-76, and Vav-1 all play an important role in the activation of PKC $\theta$  (Herndon et al., 2001; Villalba et al., 2000). Following TCR ligation, WIP phosphorylation

Table 1. Translocation of CrkL, WIP, and WASP to the T-APC Interface

Cells	% of Conjugates with Accumulation at the T Cell-APC Interface					
	CrkL		WIP		WASP	
SEE:	-	+	-	+	-	+
Wild-type Jurkat	8.5 ± 5.7	26.5 ± 5.0	15.0 ± 1.2	36.0 ± 2.3	8.0 ± 2.2	34.0 ± 2.4
P116/vector	7.5 ± 3.4	10.5 ± 5.7*	13.0 ± 1.2	13.5 ± 1.9*	4.0 ± 1.5	6.0 ± 2.3*
P116/ZAP-70 wt	8.5 ± 4.1	26.0 ± 2.3	14.0 ± 1.6	38.5 ± 1.9	6.0 ± 1.6	36.0 ± 2.0
P116/ZAP-70 KD	9.0 ± 6.0	13.0 ± 6.0*	15.5 ± 2.5	17.0 ± 4.2*	5.0 ± 2.4	9.0 ± 3.6*
CrkL/ΔSH3.1	9.5 ± 5.2	34.0 ± 5.0	12.0 ± 2.0	26.0 ± 2.5*	6.0 ± 2.4	22.0 ± 2.2*

Results represent the mean ± SD of four experiments (fifty conjugates were examined in each).

\*p < 0.01 compared to wt Jurkat cells.

resulting in loss of Ab C14 reactivity was not detectable in Jurkat T cells that lacked ZAP-70 (P116 cells) or SLP-76 (J14 cells) nor in T cells from *Vav-1*<sup>-/-</sup> mice. These results suggest that PKC $\theta$  plays an important role in the phosphorylation of WIP.

**The WASP-WIP Complex Is Disrupted following TCR Ligation**

The WASP binding region of WIP (aa 416–488) overlaps with the region recognized by C14 Ab (aa 483–496). We considered the possibility that WIP phosphorylation after TCR ligation may result in the dissociation of WASP from WIP, which would allow its activation by Cdc42. In fact, we had observed decreased amounts of WIP

in WASP immunoprecipitates from anti-CD3-stimulated Jurkat cells (Figure 2B). To confirm this result, WIP immunoprecipitates were prepared from anti-CD3-stimulated Jurkat cells using C45 Ab and were probed for WASP using a polyclonal anti-WASP antibody that recognizes the C-terminal 18 aa of WASP. Anti-CD3 stimulation resulted in a marked decrease in the association of WASP with WIP (Figure 6A). Similar results were obtained using a mAb that recognizes the WASP GBD domain and with peripheral blood T cells (data not shown). It is unlikely that the decreased WASP signal is due to failure of the antibodies used to recognize WASP, which may have been phosphorylated following TCR ligation, because treatment of WIP immunoprecipitates

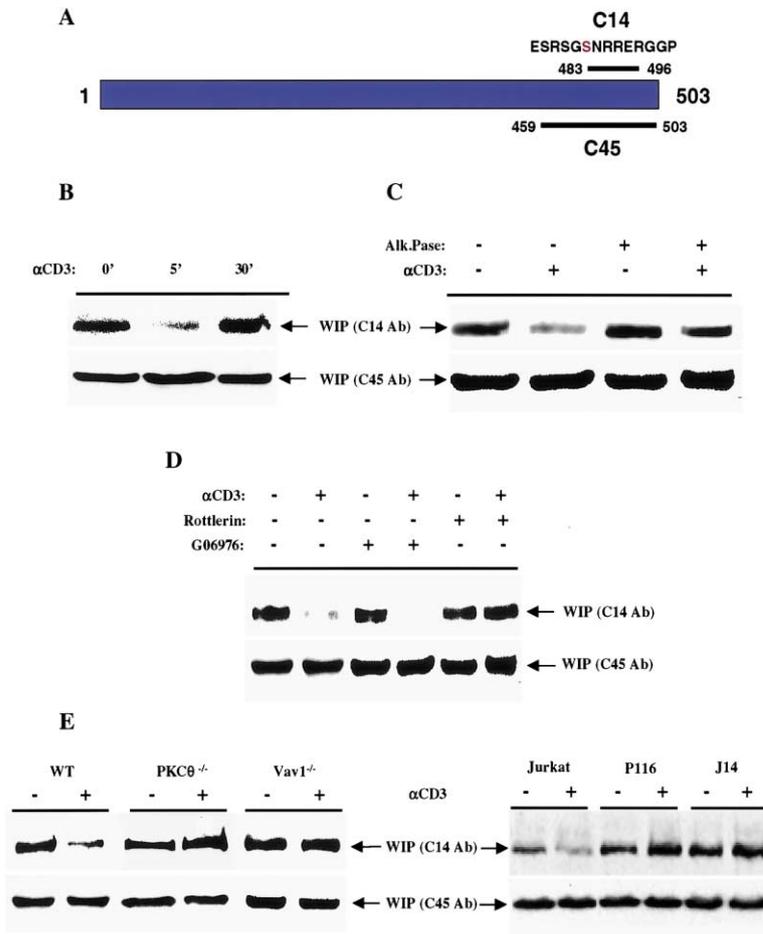


Figure 5. WIP Is Phosphorylated after TCR Ligation by PKC $\theta$

(A) Map showing WIP peptides used to raise C14 and C45 Abs.  
 (B) Reactivity of lysates from Jurkat cells before and after anti-CD3 stimulation (5 and 30 min) with C14 and C45 Abs.  
 (C) Reversal of loss of C14 Ab reactivity after anti-CD3 stimulation (5 min) by treatment of lysates with alkaline phosphatase.  
 (D) Effect of the PKC inhibitors on loss of C14 Ab reactivity after anti-CD3 stimulation (5 min). Cells were pretreated with 20  $\mu$ M rottlerin or 62 nM G06976 for 30 min at 37°C.  
 (E) Role of PKC $\theta$ , Vav-1, ZAP-70, and SLP-76 in WIP phosphorylation after TCR ligation. Splenic T cells from PKC $\theta$ <sup>-/-</sup> and Vav-1<sup>-/-</sup> mice and from wt Jurkat T cells, P116 cells, and J14 cells were stimulated with anti-CD3 for 5 min, lysed, and probed with C14 and C45 Abs.

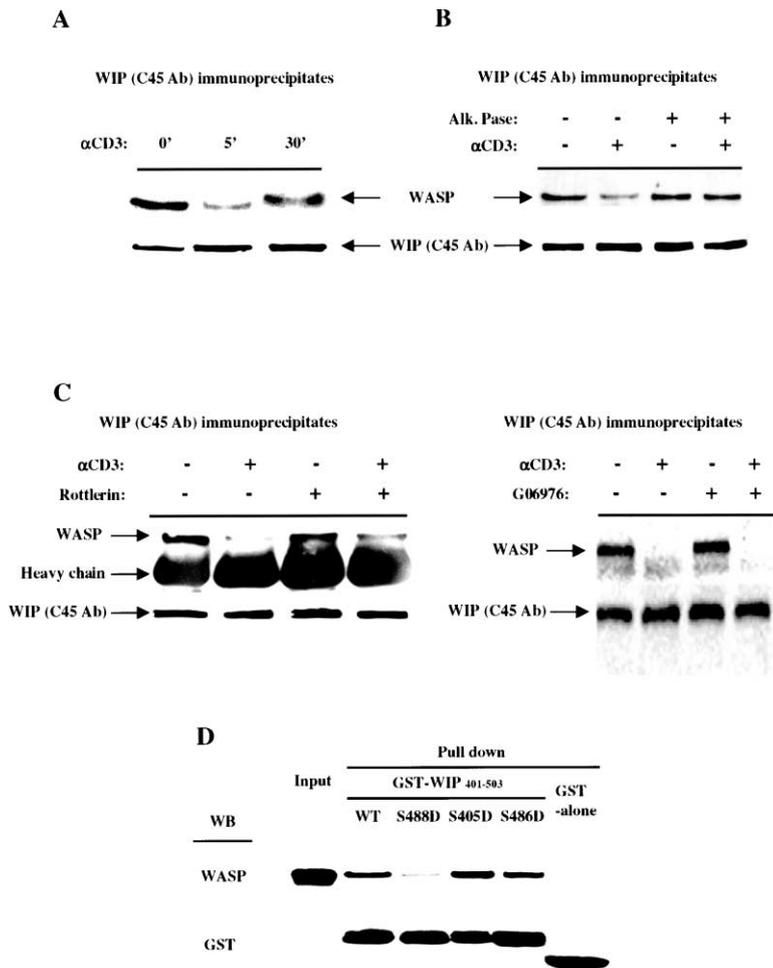


Figure 6. WIP Dissociation from WASP following TCR Ligation

(A) Dissociation of WASP from WIP following anti-CD3 stimulation (5 and 30 min) of Jurkat cells. WIP C45 Ab immunoprecipitates were probed for WASP and WIP as loading controls.

(B) Reversal of the dissociation of WIP and WASP by treatment of cell lysates with alkaline phosphatase, 1 U/10 μl at 25°C for 3 hr. (C) Effect of pretreatment with rottlerin (left panel) and G06976 (right panel) on the dissociation of WIP and WASP after TCR ligation (5 min). In all cases, WIP C45 Ab immunoprecipitates from Jurkat cells were probed with WASP polyclonal Ab and WIP C45 Ab as loading control.

(D) Pull-down assay using wt GST-WIP<sub>401-503</sub> fusion protein and its mutants S405D, S486D, and S488D with in vitro transcribed and translated WASP. Bound proteins were eluted, run on SDS-PAGE, and Western blotted for WASP and GST as loading controls.

with alkaline phosphatase did not increase the intensity of the WASP signal (data not shown). It is also unlikely that the decreased association of WASP with WIP was due to degradation of WASP, because the intensity of the WASP band in cell lysates remained unchanged after TCR ligation, and no smaller molecular weight bands that react with anti-WASP were detected (data not shown).

We next investigated the role of phosphorylation in the dissociation of the WIP-WASP complex following TCR ligation. Treatment of cell lysates with alkaline phosphatase prior to immunoprecipitation restored the association of WASP with WIP (Figure 6B). Since WIP is a target for PKC $\theta$  phosphorylation, we examined the effect of PKC inhibitors on the dissociation of the WIP-WASP complex. Figure 6C shows that pretreatment of Jurkat cells with rottlerin partially inhibited this dissociation, whereas G06976 had no effect. These results suggest that WIP phosphorylation by PKC $\theta$  results in dissociation of the WIP-WASP complex.

S488 is the only residue in WIP that falls within a consensus motif for PKC phosphorylation. We therefore examined the effect of an S488→D substitution, which mimics the negative charge of phosphoserine, on the ability of WIP to bind WASP. Since full-length WIP is poorly expressed in bacterial systems, we constructed a GST-WIP<sub>401-503</sub> fusion protein that contains the WASP

binding site (aa 416–488) as well as the point mutants S488D, S405D, and S486D. Affinity precipitation using recombinant WASP showed that wt WIP<sub>401-503</sub> bound to WASP. The S488D mutant, but not the S405D and S486D mutants, showed markedly decreased WASP binding (Figure 6D). These results suggest that phosphorylation at S488 disrupts WIP binding to WASP.

#### Activation of PKC Is Essential for F-Actin Accumulation and IL-2 Synthesis, but Not for WASP Translocation to the T Cell-APC Interface

To test the hypothesis that WASP-mediated F-actin polymerization may be dependent on PKC phosphorylation of WIP and the subsequent release of WASP from WIP inhibition, we examined F-actin accumulation at the T cell-APC interface in Jurkat cells pretreated with PKC inhibitors. F-actin accumulation at the interface was diminished in cells pretreated with rottlerin, but not in cells pretreated with G06976 (Figure 7A). In contrast, accumulation of CrkL and WIP and WASP was not affected. Since SLP-76 is essential for PKC $\theta$  activation, we also examined SLP-76-deficient J14 cells. CrkL and WIP accumulated normally at the interface in these cells. However, the amounts of WASP and F-actin that accumulated at the interface were diminished, although the percentage of conjugates with accumulation of WASP

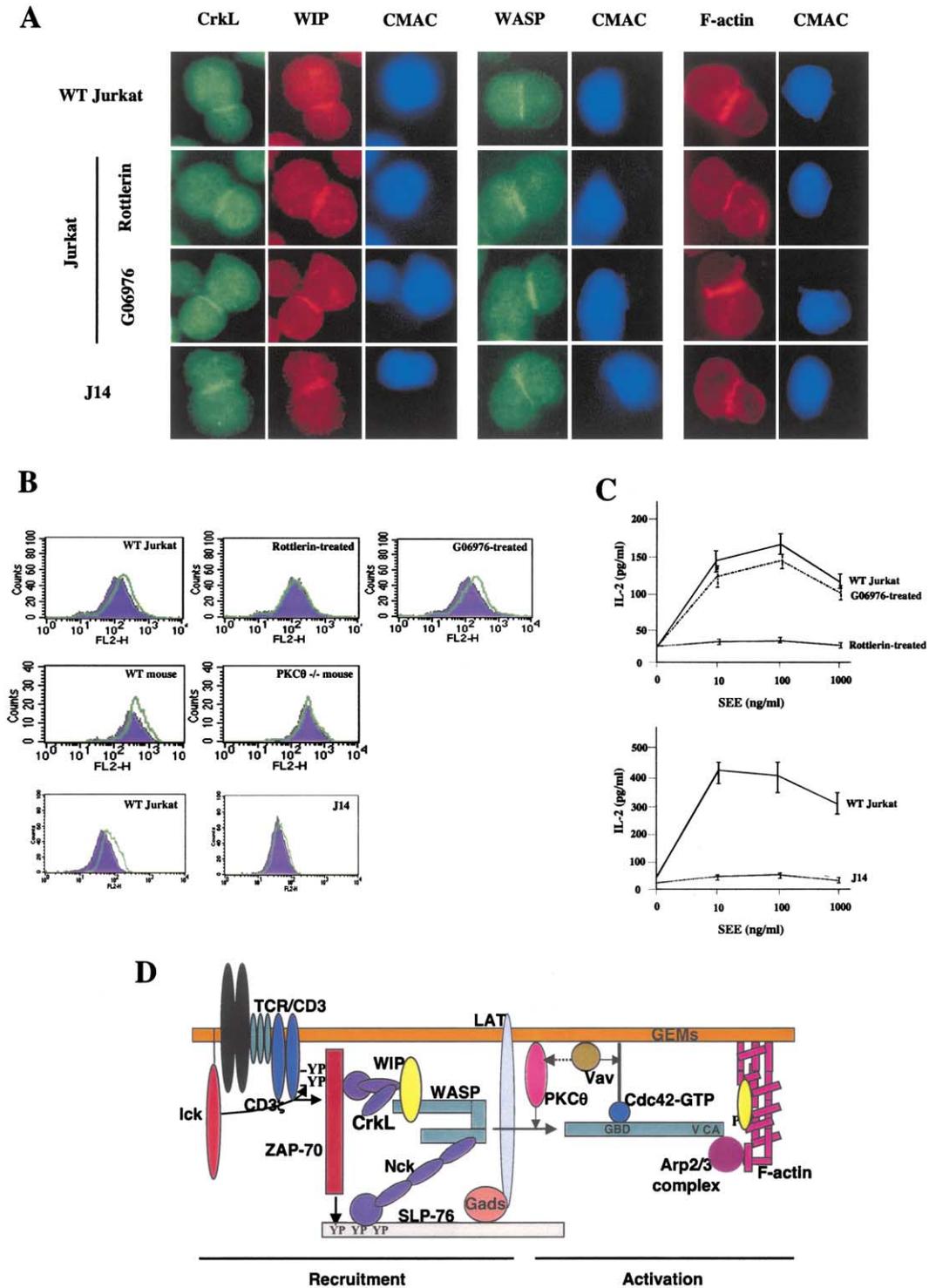


Figure 7. Effects of PKC Inhibitors and SLP-76 on the T Cell-APC interface, F-Actin Content, and IL-2 Secretion

(A) Effect of PKC inhibitors and of SLP-76 deficiency (J14 cells) on the accumulation of CrkL, WIP, WASP, and F-actin at the IS. Magnification is 600 $\times$ .

(B) F-actin content following TCR ligation in wt Jurkat cells untreated or pretreated with rottlerin or G06976, splenic T cells from wt and PKC $\theta^{-/-}$  mice, and J14 cells were analyzed by FACS. Representative results of three independent experiments are shown.

(C) Effect of PKC inhibitors (upper panel) and SLP-76 (lower panel) on IL-2 secretion by Jurkat T cells or J14 cells stimulated with SEE and Raji B cells. Means  $\pm$  SD of three independent experiments are shown.

(D) Model for the recruitment of WASP to GEMs and the IS and for its activation that results in actin polymerization following TCR ligation.

and F-actin at the interface was unaffected. All cells made normal numbers of conjugates (data not shown).

We next investigated the role of PKC $\theta$  and SLP-76 in the increase in total F-actin content after TCR ligation. Pretreatment with rottlerin, but not G06976, inhibited F-actin increase in anti-CD3-stimulated Jurkat cells. More importantly, anti-CD3-stimulated F-actin increase was severely diminished in T cells from PKC $\theta^{-/-}$  mice. Furthermore, it was also severely diminished in SLP-76-deficient J14 cells. (Figure 7B and Supplemental Figure S2 at <http://www.molecule.org/cgi/content/full/10/6/1269/DC1>).

F-actin polymerization is critical for IL-2 synthesis after TCR ligation. Pretreatment with rottlerin, but not G06976, severely impaired IL-2 secretion by Jurkat cells stimulated with SEE presented by APCs (Figure 7C). Similarly stimulated J14 cells were also severely impaired in their ability to secrete IL-2, in agreement with data obtained using anti-CD3 stimulation (Yablonski et al., 1998). Taken together with previous data showing impaired IL-2 production by T cells from PKC $\theta^{-/-}$  mice, these results suggest that SLP-76-dependent activation of PKC $\theta$  is essential for WASP-dependent F-actin polymerization and IL-2 synthesis, but not for the recruitment of the CrkL-WIP-WASP complex to the interface.

## Discussion

The results of this study provide evidence for a mechanism of WASP recruitment to the T cell-APC interface and activation that involves recruitment by ZAP-70 of a CrkL-WIP-WASP complex followed by PKC $\theta$ -mediated phosphorylation of WIP and subsequent release of WASP from WIP inhibition.

CrkL was shown to directly interact with WIP, but not with WASP. The CrkL and the WASP binding sites on WIP do not overlap. It is likely that the three proteins form a complex in cells, because CrkL was detected in WASP immunoprecipitates from unstimulated Jurkat cells (Figure 2B). Furthermore, WASP has been detected in CrkL immunoprecipitates from platelets (Oda et al., 2001). Following TCR ligation, CrkL, WIP, and WASP were found to associate with ZAP-70. Association of CrkL with ZAP-70 is probably direct, because a GST-CrkII fusion protein binds phosphorylated ZAP-70 in a Far Western assay (Gelkop and Isakov, 1999). Direct binding of CrkL to ZAP-70 would be consistent with the presence of a consensus CrkL SH2 domain binding sequence pYxxP in the interdomain B region of ZAP-70 (Chan et al., 1992). Therefore, it is likely that the order of association is ZAP-70-CrkL-WIP-WASP.

The formation of a ZAP-70-CrkL-WIP-WASP complex provides a mechanistic basis for our observation that WASP is recruited together with WIP and CrkL to GEMs. Phosphorylated ZAP-70 is recruited to GEMs by the CD3 $\zeta$  subunit of the TCR complex and was found to be essential for the translocation of WASP, WIP, and CrkL to GEMs. The kinase activity of ZAP-70 is critical for this translocation, suggesting that autophosphorylation of ZAP-70 generates the phosphotyrosine(s) that is essential for CrkL docking. Further proof of the importance of the ZAP-70-CrkL-WIP-WASP complex in the recruitment of WASP to GEMs was provided by the observation

that expression of a dominant-negative CrkL mutant that fails to bind WIP but retains ZAP-70 binding capacity inhibited the recruitment of WASP and WIP to GEMs.

WASP, WIP, and CrkL all localized in the IS following TCR ligation. Our findings suggest that the ZAP-70-CrkL-WIP-WASP complex plays a role in this recruitment, since it required ZAP-70 kinase activity and since expression of dominant-negative CrkL partially inhibited the recruitment of WASP and WIP to the IS. The functional importance of the ZAP-70-CrkL-WIP recruitment pathway is supported by the observation that F-actin accumulation at the T cell-APC interface, total cellular F-actin content increase, and IL-2 synthesis following TCR ligation were all diminished in ZAP-70-deficient cells and in cells that expressed dominant-negative CrkL, and are also impaired in WIP $^{-/-}$  T cells (Anton et al., 2002).

Our studies show that following TCR ligation, WIP is phosphorylated by PKC $\theta$ , and the WIP-WASP complex dissociates. WIP phosphorylation was strongly suggested by loss of reactivity with an antibody that recognizes a peptide that contains a PKC target motif and by the recovery of this reactivity following treatment with alkaline phosphatase. The role of PKC $\theta$  in WIP phosphorylation is suggested by its inhibition by rottlerin, a PKC $\delta$  and  $\theta$  inhibitor, and was confirmed using T cells from PKC $\theta^{-/-}$  mice. Consistent with previous studies showing that ZAP-70, SLP-76, and Vav-1 are important for PKC $\theta$  activation (Herndon et al., 2001; Villalba et al., 2000), these molecules were found to be important for WIP phosphorylation.

Phosphorylation of the WIP-WASP complex following TCR ligation was clearly responsible for its dissociation, because treatment of the complex with alkaline phosphatase allowed it to re-form. Both tyrosine kinases and serine/threonine kinases are activated after TCR ligation. However, WIP is not tyrosine phosphorylated after TCR ligation (data not shown), and tyrosine phosphorylation of WASP has not been reported in T cells. Dissociation of the WIP-WASP complex was selectively inhibited by rottlerin, suggesting the involvement of a nonclassical PKC. It is likely that PKC phosphorylation of WIP is critical for WASP dissociation because WASP contains no conserved target sequences for PKC. In contrast, WIP contains a single PKC phosphorylation motif surrounding S488 and located at the C-terminal end of the WASP binding region of WIP. Substitution of S488 $\rightarrow$ D markedly reduced WIP binding to WASP. Taken together, the data suggest that PKC $\theta$  phosphorylation of WIP at Ser488 disrupts the WIP-WASP complex. We cannot rule out a role for the phosphorylation of other residues of WIP or WASP in the dissociation of the WIP-WASP complex.

Phosphorylation-dependent dissociation of the WIP-WASP complex had important functional implications for F-actin polymerization and T cell activation following TCR ligation. Selective inhibition of nonclassical PKCs by rottlerin, as well as lack of SLP-76, which is essential for PKC activation, resulted in impairment of F-actin accumulation at the T cell-APC interface, total cellular F-actin increase and IL-2 secretion. More importantly, T cells from PKC $\theta^{-/-}$  mice failed to increase their F-actin content following TCR ligation. This finding, together with the previously reported impaired IL-2 production

by these cells (Sun et al., 2000), suggests that PKC $\theta$  is important for WASP-dependent F-actin polymerization and IL-2 synthesis.

WASP recruitment to the interface was not affected by inhibition of nonclassical PKCs, but was partially decreased in SLP-76-deficient J14 cells. In contrast, recruitment of CrkL and WIP was not affected in J-14 cells, consistent with our finding that these two proteins associate normally with ZAP-70 in these cells (data not shown). These results suggest that SLP-76 plays a role in WASP localization at the interface. This is possibly mediated by the formation of a SLP-76-Nck-WASP complex following TCR ligation (Krause et al., 2000) and the recruitment of this complex by Gads to LAT, which is localized in GEMs and at the interface (Tamir et al., 2000). This mechanism of WASP recruitment may underlie the previously reported role of the polyproline-rich region of WASP in its recruitment to the IS (Cannon et al., 2001), since this region binds Nck. The recent observation that Nck binds directly to CD3 $\epsilon$  after TCR ligation (Gil et al., 2002) suggests yet another potential mechanism of WASP recruitment to the IS.

Failure to recruit WASP to ZAP-70 may explain the failure of WIP $^{-/-}$  T cells to increase F-actin following TCR ligation (Anton et al., 2002) and the observation that missense point mutations in WASP that affect WIP binding result in WAS (Stewart et al., 1999). However, the role of WIP may not be restricted simply to recruiting WASP to ZAP-70. WIP binds and stabilizes actin filaments and thus may help stabilize the IS. Stabilization of actin filaments may explain the observation that over-expression of WIP causes an increase in total cellular F-actin and that WIP deficiency is associated with a disorganized actin network in lymphocytes (Anton et al., 2002; Ramesh et al., 1997). In addition, WIP may also participate independently of WASP in actin polymerization, since its yeast homolog, verprolin, is important for actin polymerization by the myosins Myo3p and Myo5p (Geli et al., 2000).

TCR ligation results in Lck phosphorylation of CD3 $\zeta$  and recruitment of ZAP-70 to the TCR and in the translocation of the TCR/ZAP-70 complex to GEMs. Our results support the following model that links TCR activation to actin polymerization (Figure 7D). Phospho-ZAP-70 recruits a preformed CrkL-WIP-WASP complex to GEMs and to the IS. In GEMs, WIP is phosphorylated by PKC $\theta$ , the activation of which is dependent on Lck, Vav-1, ZAP-70, and SLP-76. WASP disengages from the phosphorylated WIP, releasing it from inhibition and allowing it to be activated by membrane-bound Cdc42 and to initiate Arp2/3 complex-dependent actin polymerization. WIP binds to newly formed F-actin and may help stabilize the IS, facilitating the activation of signaling intermediates and transcription factors that are needed for IL-2 gene expression and T cell proliferation. Our model does not exclude alternate pathways of recruitment of WASP to the IS, e.g., via SLP-76 and Nck. Further work is needed to assess the relative contribution of various WASP recruitment pathways to T cell activation.

#### Experimental Procedures

##### Cells, Mice, Reagents, and Antibodies

Jurkat E6-1 T cells and Raji B cells were obtained from ATCC. P116 (ZAP-70 deficient) cells were established as described previously

(Abraham, 2000), and J14 (SLP-76 deficient) cells were a kind gift of Dr. A. Weiss (UCSF). Cells were cultured in RPMI-1640 medium with 10% fetal calf serum. T cell blasts were established from human PBMCs by stimulation with 5  $\mu$ g/ml PHA and 10 U/ml IL-2 for 7 days. PKC $\theta^{-/-}$  mice are described in Sun et al. (2000). Vav-1 $^{-/-}$  mice were a gift of Dr. Fred Alt (Zhang et al., 1995). Mouse splenic T cells were purified as previously described (Anton et al., 2002).

The PKC inhibitors rottlerin and G06976 were from Calbiochem. Alkaline phosphatase was from Roche Diagnostics. Rabbit anti-WIP C45 and C14 antisera were raised against peptides 459–503 and 483–496, respectively. Rabbit anti-WASP Ab (a gift of Dr. I. Molina, Univ. of Granada, Spain) was raised against the C-terminal 18 aa. Monoclonal Abs to actin (Sigma), Xpress (Invitrogen), CrkL (Upstate), CD3 $\zeta$ , (BD PharMingen), phosphotyrosine (PY20), ZAP-70, and PKC $\theta$  (all from Transduction Laboratories), and WASP (Kawai et al., 2002) were used.

##### Protein Preparation and Pull-Down Assay

Xpress-tagged full-length WIP protein was prepared as described previously (Martinez-Quiles et al., 2001). Linearized human WASP cDNA (1  $\mu$ g) was in vitro transcribed and translated with 50  $\mu$ l of TNT-coupled reticulocyte lysate system (Promega). Full-length CrkL and CrkL cDNAs in pGEX vector were gifted by Dr. B. Mayer (Univ. of Connecticut). GST fusion proteins were induced, purified, and used in pull-down assays as described previously (Anton et al., 1998), using 2  $\mu$ g GST fusion proteins or GST alone with 20  $\mu$ l glutathione-sepharose beads for 2  $\mu$ g Xpress-tagged WIP or WASP. Bound proteins were eluted by boiling in Laemmli buffer for 5 min, run on SDS-PAGE gels, and analyzed by Western blotting.

GST-WIP<sub>401-503</sub> containing no mutation (wt) and S405 $\rightarrow$ D, S486 $\rightarrow$ D, and S488 $\rightarrow$ D mutants were generated by PCR using the appropriate oligonucleotides in which the relevant Ser codons are replaced with Asp. PCR amplicons were verified by DNA sequencing and cloned into pGEX-6P1 in-frame with GST. The induced fusion proteins were purified and used in pull-down assays.

##### Yeast Two-Hybrid System

Full-length or deletion mutants of human WASP and WIP were cloned in-frame in the bait vector pGBT9 and activation domain vector pGAD424 (Clontech), respectively, as previously described (Ramesh et al., 1997). Full-length CrkL (aa 1–304) or the CrkL deletion mutants  $\Delta$ SH2 (del. aa 1–111),  $\Delta$ SH3.2 (del. aa 199–304),  $\Delta$ SH2+ $\Delta$ SH3.1 (del. aa 1–215),  $\Delta$ SH2+ $\Delta$ SH3.2 (del. aa 1–111 and 199–304), and  $\Delta$ SH3.1+ $\Delta$ SH3.2 (del. aa 95–304) of human CrkL were cloned in-frame in both vectors. All constructs were verified by sequencing. Double transfectants were selected on Leu $^{-}$ Trp $^{-}$  media, and blue color at 4 hr resulting from  $\beta$ -galactosidase activity was observed.

##### Cell Stimulation, Immunoprecipitation, and Western Blotting

Human T cells were incubated with 10  $\mu$ g/ml mouse anti-CD3 $\epsilon$  mAb, UCHT1 (Calbiochem), on ice for 30 min then with 15  $\mu$ g/ml goat anti-mouse IgG(H+L) (Caltag) for 3–30 min at 37°C. For mouse T cells, rat anti-mouse CD3 mAb KT3 (Serotec) and goat anti-rat IgG (ICN Pharmaceuticals) were used.

Cells were lysed in ice-cold lysis buffer containing 1% Triton X-100, and lysates were centrifuged for 15 min at 4°C and precleared for 1 hr at 4°C with protein G sepharose (Amersham Pharmacia Biotech). Immunoprecipitation was performed overnight at 4°C with antibody (4  $\mu$ g) or antiserum (4  $\mu$ l) preadsorbed onto protein G sepharose. Beads were washed five times with modified lysis buffer containing 0.2% Triton X-100. Bound proteins were eluted, run on SDS-PAGE 4%–15% gradient gels, and analyzed by Western blotting with the indicated antibodies followed by goat anti-mouse or rabbit antibodies conjugated to horseradish peroxidase (HRP) and enhanced chemiluminescent detection (ECL) (PerkinElmer).

##### Stable Transfection

Full-length CrkL and  $\Delta$ SH3.1 CrkL were cloned in pcDNA3.1 vector (Invitrogen) and verified by sequencing. Myc-tagged wild-type ZAP-70 and kinase-dead ZAP-70 with a missense mutation in the kinase domain (K69R) in pcDNA3 were described previously (Williams et al., 1998). 1  $\mu$ g of linearized plasmids was transfected by electropor-

ation into  $1 \times 10^7$  Jurkat or P116 cells using Bio-Rad GenePulser II at 250V, 975  $\mu$ F. Cells were selected with 600–800  $\mu$ g/ml of G418 (Calbiochem) for 14 days. Selected cells were subjected to limiting dilution as described previously (Ramesh et al., 1997), and clones were isolated.

#### Lipid Rafts Isolation

Lipid raft fractions were prepared as described (Zhang et al., 1998) with modifications. Lysates of  $5 \times 10^7$  cells in 1 ml lysis buffer containing 0.5% Triton X-100 were homogenized in a dounce homogenizer, diluted with 1 ml of 80% sucrose in TNE buffer, layered at the bottom of Beckman 13  $\times$  51 mm centrifuge tubes, and overlaid with 2 ml of 30% sucrose and then 1 ml of 5% sucrose. Sucrose gradients were centrifuged at  $200,000 \times g$  for 16–18 hr at 4°C, and twelve 0.4 ml fractions were collected starting from the top of the gradient. Equal volumes of each fraction or of pooled fractions 2–5 were subjected to Western blotting. For GM1 analysis, they were slit blotted and incubated with biotin-labeled cholera toxin B (Sigma) followed by streptavidin-peroxidase conjugate (Sigma) and ECL detection.

#### T Cell-APC Conjugate Formation and Immunofluorescence Microscopy

Raji B cells were labeled with 10  $\mu$ M blue fluorescent cell tracker CMAC (Molecular Probes) for 30 min and then incubated with 5  $\mu$ g/ml superantigen SEE (Toxin Technologies) for 30 min at 37°C. They were incubated with an equal number of T cells at 37°C for 10 min, and the mixture was plated onto poly-L-lysine (Sigma)-coated coverslips. Conjugates were scored visually. Intracellular immunofluorescence was performed as described (Martinez-Quiles et al., 2001), using affinity-purified rabbit anti-WIP Ab or mAbs to WASP and CrkL followed by TRITC-labeled goat anti-rabbit secondary antibody or Alexa green-labeled goat anti-mouse antibody (Sigma), and 1  $\mu$ g/ml TRITC-phalloidin (Sigma) for F-actin staining. Fifty T:B conjugates were examined, and those showing a distinct band of labeling at the contact site were scored.

#### Determination of Cellular F-Actin Content and IL-2 Secretion

Cells were fixed in 4% formaldehyde, washed, permeabilized, and stained in a single step with 0.1% Triton X-100 and 5  $\mu$ g/ml TRITC-phalloidin, and F-actin content was analyzed by FACS, as described (Anton et al., 2002). For IL-2 secretion, T cells and irradiated Raji B cells ( $1 \times 10^5$  each) were cultured in 200  $\mu$ l culture medium with SEE for 24 hr or for 6 hr in the case of addition of PKC inhibitors. IL-2 in supernatants was measured by ELISA (R&D).

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