# Formation of a WIP-, WASp-, actin-, and myosin IIA–containing multiprotein complex in activated NK cells and its alteration by KIR inhibitory signaling

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The tumor natural killer (NK) cell line YTS was used to examine the cytoskeletal rearrangements required for cytolysis. A multiprotein complex weighing ~1.3 mD and consisting of WASp-interacting protein (WIP), Wiskott-Aldrich syndrome protein (WASp), actin, and myosin IIA that formed during NK cell activation was identified. After induction of an inhibitory signal, the recruitment of actin and myosin IIA to a constitutive WIP–WASp complex was greatly decreased. Both actin and myosin IIA were recruited to WIP in the absence of WASp.

This recruitment correlated with increased WIP phosphorylation, which was mediated by PKCθ. Furthermore, the disruption of WIP expression by *WIP* RNA interference prevented the formation of this protein complex and led to almost complete inhibition of cytotoxic activity. Thus, the multiprotein complex is important for NK cell function, killer cell immunoglobulin-like receptor inhibitory signaling affects proteins involved in cytoskeletal rearrangements, and WIP plays a central role in the formation of the complex and in the regulation of NK cell activity.

## Introduction

The activation of natural killer (NK) cells, which is mediated by the recognition of their ligands by activation receptors, triggers a complex, highly regulated response leading to the death of a target cell. Cytolytic responses require reorganization of the actin cytoskeleton for receptor translocation at the cell surface to facilitate conjugate formation, initiation, the continuation of signaling, and effective killing (Carpen et al., 1983; Vyas et al., 2002). This dynamic and highly complex process is regulated by a variety of actin-binding proteins, such as cofilin, profilin, and Wiskott-Aldrich syndrome protein (WASp), as well as Scar family proteins, thymosins, capping proteins, and the Arp2/3 complex (dos Remedios et al., 2003; Pollard and Borisy, 2003). The latter is crucial for actin nucleation and formation of new actin filament branches (Higgs and Pollard, 2001). The Arp2/3 complex, which is formed from seven subunits (Robinson et al., 2001; Volkmann et al., 2001), is closely regulated by WASp family proteins (Higgs and Pollard, 2001). WASp binds to the Arp2/3 complex (Machesky and Insall, 1998), increasing its affinity for ATP (Le Clainche et al., 2001). WASp activity, in turn,

is tightly controlled by a variety of adaptor and regulatory proteins (Orange et al., 2004), including the WASp-interacting protein (WIP; Ramesh et al., 1997).

NK cells are very useful for studying the regulation of cytotoxic cell activity because of the expression of the killer cell immunoglobulin-like receptors (KIR; Vilches and Parham, 2002) that are capable of inhibition of NK cell cytotoxicity (Long, 1999). A characteristic feature of inhibitory KIR molecules is a long cytoplasmic tail (76–95 amino acids) containing one or two immunoreceptor tyrosine-based inhibition motifs (ITIMs; Vely et al., 1996). Upon tyrosine phosphorylation, inhibitory KIR ITIMs serve as docking sites for the protein tyrosine phosphatases SHP-1 and -2 (Campbell et al., 1996; Olcese et al., 1996), which can dephosphorylate kinases and adaptor proteins that are involved in early events of signal transduction (Blery et al., 2000), leading to inhibition of NK cell activity. The number of proteins that are dephosphorylated is presently unknown.

In this study, we describe a multiprotein complex comprised of WIP, WASp, actin, and myosin IIA that is formed during NK cell activation, but is not formed in the presence of KIR2DL1 inhibitory signaling. The identification of this multimolecular protein complex that is regulated by activating and inhibitory signaling provides insight into understanding the cytoskeletal rearrangements and mechanisms essential for NK cytolytic activity.

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Abbreviations used in this paper: ITIM, immunoreceptor tyrosine-based inhibition motif; KIR, killer cell immunoglobulin-like receptor; MHC, major histocompatibility class; MS, mass spectrometry; NK, natural killer; WASp, Wiskott-Aldrich syndrome protein; WIP, WASp-interacting protein.

The online version of this article contains supplemental material.

# Results

## Formation of a multiprotein complex during NK cell activation

Cytolytic reactions are dependent on cytoskeletal proteins, but the orchestration of rearrangements of the cytoskeletal elements that is required for cytolysis is unclear. Physiologically contrasting activating and inhibitory signaling in NK cells were used to highlight important stages in control of cytoskeletal function. The inhibitory model was that of the YTS NK tumor cell line, into which the inhibitory receptor KIR2DL1 had been introduced (YTS/KIR2DL1), and a major histocompatibility class I (MHCI)–negative B lymphoblastoid 721.221 cell in which the KIR2DL1 ligand HLA-Cw6 had been expressed (721.221/Cw6). The activating paradigm was the cytolytic interaction between YTS/KIR2DL1 cells and 721.221 cells. NK cells, although expressing an inhibitory receptor, lyse class I MHC protein–negative targets. YTS/KIR2DL1 cells stably expressing FLAG-WIP (YTS/KIR2DL1/FLAG-WIP) were generated to define the cytoskeletal complex in cytolytic and noncytolytic interactions and to investigate whether WIP and/or WASp are targeted by KIR2DL1 inhibitory signaling.

First, YTS/KIR2DL1/FLAG-WIP cells were activated by mixing with the appropriate target cells. Immunoprecipitation
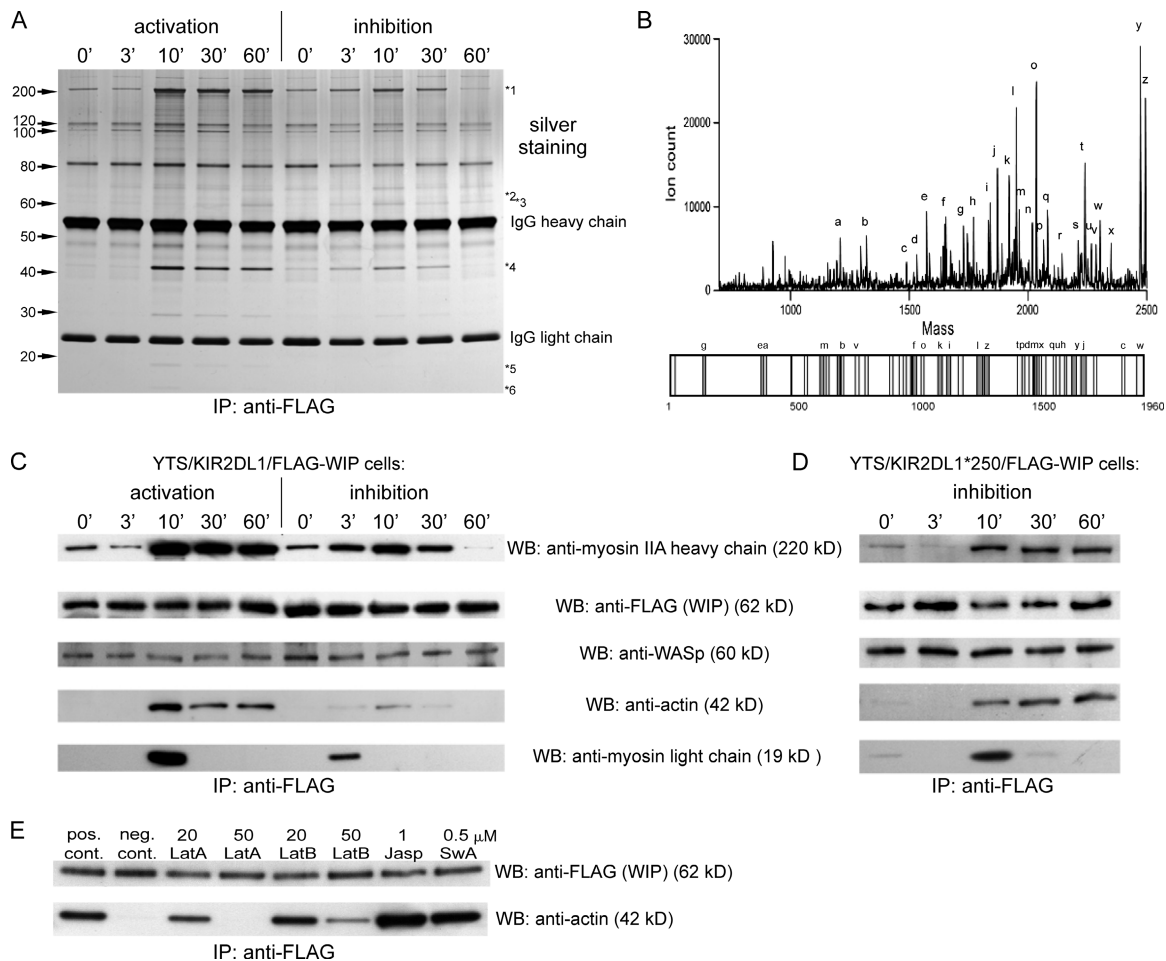


Figure 1. **A multiprotein complex formed during NK cell activation is altered by inhibitory signaling.** (A) YTS/KIR2DL1/FLAG-WIP cells were mixed with 721.221 or 721.221/Cw6 cells, immediately transferred to 37°C, and incubated for the indicated times. Cell lysates were immunoprecipitated with anti-FLAG mAb. Immunoprecipitated proteins were resolved on a 4–12% NuPage gel and stained with silver. The proteins recruited to the complex (marked by numbered asterisks) were subsequently analyzed by MS. (B) An example of vacuum matrix–assisted laser disorption ionization MS analysis of the band corresponding to the first asterisk (*1) in the silver-stained gel in A. The lettered peaks obtained were identified as matching myosin IIA heavy chain and are overlaid on a schematic of the 1,960–amino acid residue molecule (bottom). The width of the individual box is representative of the size of the given peptide. Boxes shown in dark gray were further positively identified by MS peptide sequence. (C) Western blotting analysis of the anti-FLAG–immunoprecipitated proteins shown in A. The same PVDF membrane was sequentially stripped and probed with anti–myosin IIA, anti-FLAG, anti-WASp, anti-actin, and anti–myosin light chain antibodies. The molecular masses of the proteins according to their position, relative to molecular mass markers, are shown in parentheses. (D) A KIR2DL1 mutant, lacking the ITIM motifs, is unable to affect the formation of the complex. YTS/KIR2DL1*250/FLAG-WIP cells were mixed with 721.221/Cw6 cells, immediately transferred to 37°C, and incubated for the indicated times. Cell lysates were immunoprecipitated with anti-FLAG mAb. Immunoprecipitated proteins were visualized by immunoblotting with anti–myosin IIA, anti-FLAG, anti-WASp, anti-actin, and anti–myosin regulatory light chain antibodies. (E) Effects of actin inhibitors on the interaction of WIP and actin. YTS/KIR2DL1/FLAG-WIP cells were pretreated with DMSO (controls) or with the following actin inhibitors: latrunculin A (LatA), latrunculin B (LatB), jasplakinolide (Jasp), or swinholide A (SwA) at the indicated concentrations for 30 min at 37°C, followed by mixing with 721.221 target cells (except for the negative control) for 10 min at 37°C. Cell lysates were immunoprecipitated with anti-FLAG mAb and immunoblotted with anti-FLAG and anti-actin antibodies. pos. contr., cells pretreated with 0.1% DMSO and activated by mixing with 721.221 target cells (positive control); neg. contr., resting cells treated with 0.1% DMSO (negative control).

Table I. **Main components of the multiprotein complex**

| Number | Name | Calculated molecular mass | Accession number |
|---|---|---|---|
| | | *kD* | |
| 1 | Myosin IIA | 226.5 | P35579 |
| 2 | WIP | 51.5 | O43516 |
| 3 | WASp | 52.9 | P42768 |
| 4 | Actin | 42 | P62736 |
| 5 | Myosin light chain 2 | 19.7 | P19105 |
| 6 | Myosin light chain 3 | 16.9 | P60660 |

A summary of the main components of the multiprotein complex, which were identified by tandem MS, along with their calculated molecular masses and accession numbers (available from GenBank/EMBL/DDBJ). The order numbers in the table correspond to the asterisk numbers shown in Fig. 1 A.

with anti-FLAG mAb revealed a substantial number of proteins that coimmunoprecipitated with FLAG-WIP (Fig. 1 A). Specific components of this complex (marked by asterisks in Fig. 1 A) were identified by tandem mass spectrometry (MS). The presence of WIP and WASp in the complex was confirmed, and the additional presence of actin, myosin IIA heavy chain, and two myosin light chains was revealed (Table I). An example is shown for myosin IIA identification in Fig. 1 B. The presence of these proteins was subsequently verified by immunoblotting (Fig. 1 C). Because WIP has been shown to interact with WASp (Ramesh et al., 1997), and actin has been demonstrated to bind both WIP (Martinez-Quiles et al., 2001) and WASp (Miki and Takenawa, 1998), the presence of WASp and actin in the complex was not surprising. However, the inducible recruitment of actin and the constitutive presence of WASp in the complex are

noteworthy. The presence of both heavy and light chains of myosin IIA (Fig. 1, A [left] and C) indicates their probable involvement in cytotoxic NK cell activity. An interaction between WIP and a myosin in mammalian cells has not been previously reported.

Treatment of cells with latrunculin A and B, which bind to monomeric actin and prevent its polymerization (Spector et al., 1999), eliminated actin binding to WIP in a dose-dependent manner (Fig. 1 E), as well as myosin IIA binding (unpublished data); therefore, the complex could not be formed. However, either stabilization of actin filaments by jasplakinolide or treatment of cells with swinholide A, which has filament-severing ability (Spector et al., 1999), both resulted in increased actin binding to WIP (Fig. 1 E). Thus, the actin found in the complex appears to be F-actin. The WIP–WASp interaction was stable under all conditions.

## The multiprotein complex formed during NK cell activation is >1 mD

The results implied that activation of NK cells might trigger formation of a large oligomeric structure. To test this, the relative elution positions of WIP and WASp in gel filtration chromatography in resting and activated YTS/KIR2DL1/FLAG-WIP cells were investigated.

In resting NK cells, FLAG-WIP and the majority of WASp were eluted with the partition coefficient $K_{av} = 0.43$ (Fig. 2 A), corresponding to a mass of $\sim$350–400 kD on the calibration curve. WIP and WASp migrated on SDS-PAGE as proteins of 60 and 62 kD, respectively. The presence of WIP and WASp in the relatively high mass fraction may reflect either their interaction with other proteins in the cell or oligomerization. Importantly,



$Kav_{WASp+WIP} = 0.43 \rightarrow \sim$350 - 400 kD

$Kav_{WASp+WIP} = 0.3 \rightarrow \sim$1200 - 1500 kD

resting cells

8  9  10 11 12 13 14 15 16 17 18 19 20 21

WB: anti-MyoIIA (220 kD)
WB: anti-FLAG (WIP) (62 kD)
WB: anti-WASp (60 kD)
WB: anti-actin (42 kD)

activated cells
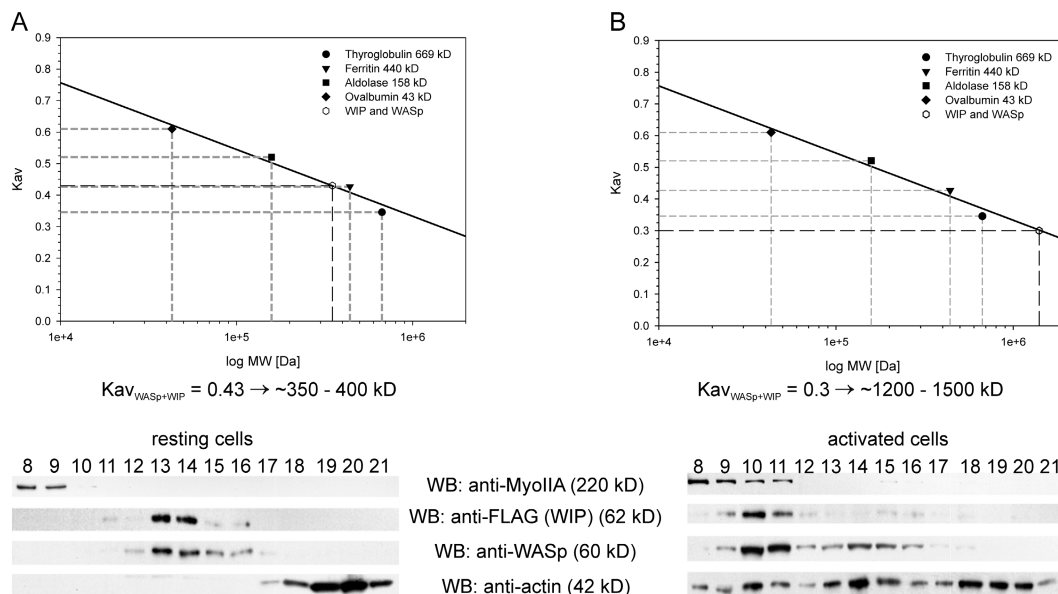
8  9  10 11 12 13 14 15 16 17 18 19 20 21

Figure 2. **The multiprotein complex formed during NK cell activation is ~1,300 kD.** YTS/KIR2DL1/FLAG-WIP cells alone or mixed with 721.221 target cells for 10 min at 37°C were lysed, and lysates were applied to the Superose 6 column. Proteins from fractions were resolved on a NuPage gel, transferred to PVDF membrane, and sequentially immunoblotted for the presence of actin, myosin IIA, WASp, and FLAG-WIP to identify the main components of the complex (only relevant fractions are shown). The molecular masses of the proteins according to their position, relative to molecular masses markers, are shown in parentheses. $K_{av}$ values for proteins of interest were calculated based on elution volumes and fitted to a calibration curve to estimate the size of complexes. (A) In nonstimulated NK cells, WIP and WASp exist as a part of a complex of ~350–400 kD, and neither myosin IIA nor actin are present in the fractions containing WIP and WASp. (B) After NK cell stimulation, WIP and WASp, along with actin and myosin IIA, become part of a multimeric complex with a mass of ~1,200–1,300 kD.

neither myosin IIA nor actin was found in the fractions containing WIP and WASp, demonstrating that the complex was not formed in resting cells. However, the activation of NK cells resulted in the shift of the majority of WIP and WASp to a fraction, with the partition coefficient $K_{av} = 0.3$, corresponding to a mass of $\sim$1,200–1,500 kD (Fig. 2 B). Moreover, the change of WIP–WASp position was accompanied by the simultaneous appearance of myosin IIA and actin in the same fraction, indicating that a multiprotein complex of $\sim$1,300 kD was formed after NK cell activation.

The majority of actin in both resting and activated cells, likely representing the monomeric actin pool, was eluted with $K_{av} = 0.61$ (Fig. 2), which is the same $K_{av}$ as that of the molecular mass standard ovalbumin (43 kD), thus validating the measurements.

## KIR2DL1 signaling affects complex formation

To evaluate the function of inhibitory receptor KIR2DL1 in YTS cells conjugated with physiological target cells, phosphorylation of the receptor was investigated. First, phosphorylation of KIR2DL1 that had previously been transduced into YTS cells (Cohen et al., 1999) after antibody cross-linking was determined over time. Maximum KIR2DL1 phosphorylation in YTS cells was found at 3 min (Fig. 3), in agreement with a previous study (Faure et al., 2003) showing maximum phosphorylation of KIR at 3.5 min after ligand binding on the surface of insect cells. Induction of KIR2DL1 phosphorylation was specific because irrelevant antibody did not induce KIR phosphorylation. Importantly, a signaling-deficient KIR2DL1*250 mutant lacking both ITIMs (Fassett et al., 2001) was not phosphorylated. Interestingly, KIR2DL1 migrated on the gel as a doublet after

induction, likely because of differential phosphorylation of the two ITIMs in KIR2DL1 because at time 0 unphosphorylated KIR2DL1 migrated as a single band. Similarly, KIR2DL1*250 migrated as a single nonphosphorylated band (Fig. 3).

Next, the effect of inhibitory signaling in YTS/KIR2DL1/FLAG-WIP cells was examined using 721.221/Cw6 cells as the target, as HLA-Cw6 is a ligand for KIR2DL1 (Colonna et al., 1993). Complex formation was strongly diminished in the presence of KIR2DL1-mediated signaling (Fig. 1, A [right] and C). Specifically, actin was recruited to the complex after 3 min of NK cell activation, with maximum phosphorylation at 10 min, and its amount decreased with time. In contrast, inhibitory signaling largely prevented the appearance of actin in the complex. Association of myosin IIA with the WIP–WASp complex increased substantially by 10 min after NK cell activation and correlated with the appearance of myosin regulatory light chain and actin. An increased amount of myosin IIA was observed, even at 60 min. In contrast, NK cell inhibition only resulted in a slight increase in the amount of myosin IIA and its association with the WIP-based complex was quickly disrupted. Thus, KIR2DL1 inhibitory signaling effected the interaction of myosin IIA with the complex, similarly to actin. Interestingly, WASp that is present in FLAG-WIP immunoprecipitates was not affected by inhibitory signaling.

To test if the observed effects were KIR2DL1 mediated, the YTS/KIR2DL1*250 cell line that is deficient in KIR signaling was used. The binding of KIR2DL1*250 to its ligand had no effect on complex formation (Fig. 1 D), and the resulting complex was identical to that found in activated YTS/KIR2DL1 cells. Thus, KIR2DL1*250 was unable to influence complex formation, demonstrating that the KIR inhibitory receptor signal affects complex assembly and/or function of the multiprotein complex created during NK cell activation.

## WIP, WASp, and myosin IIA polarize to the cell–cell contact site with F-actin after NK cell activation

Formation of the complex and its alteration by inhibitory signaling raised the question of whether or not the complex is a part of the immune synapse. Therefore, we examined the localization of the complex in the cell after activation or inhibition. In resting YTS/KIR2DL1/FLAG-WIP cells, nearly all FLAG-WIP colocalized with WASp, but not all WASp colocalized with FLAG-WIP, likely reflecting a pool of WASp either bound to endogenous WIP or not associated with WIP (Fig. 4 A). Importantly, both proteins were dispersed throughout the cytoplasm, whereas myosin IIA (as well as F-actin; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200509076/DC1) was located at the cell periphery. The activation of NK cells by mixing with target cells resulted in the relocation of FLAG-WIP and WASp and the accumulation of myosin IIA at the cell–cell contact site (Fig. 4 A), where F-actin was also localized (Fig. S1; Orange et al., 2003). FLAG-WIP was polarized toward the contact site in 76% of conjugates of NK cells with susceptible target cells, and 16% of it was found at the synapse. Similarly, WASp was polarized in 64% of cytolytic conjugates, with 22% found at the cell–cell interface. The remainder
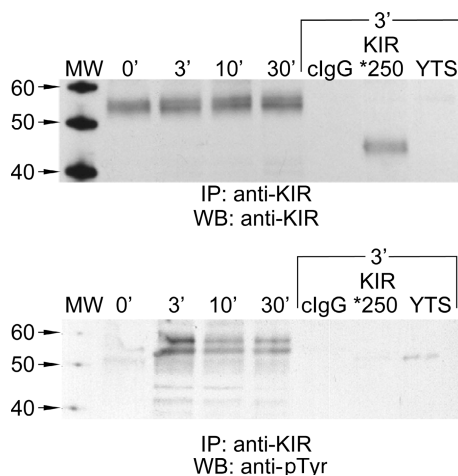


Figure 3. **KIR2DL1 phosphorylation.** YTS cells alone or transfected with KIR2DL1 or KIR2DL1*250 (Fassett et al., 2001) were incubated for the indicated times at 37°C with protein G beads coated with anti-KIR2DL1 mAb or control IgG (cIgG; YTS/KIR2DL1 cells only). Immunoprecipitated material was immunoblotted with anti-KIR2DL1 polyclonal antibodies (top) or anti-phosphotyrosine mAb (bottom). The double band present in both images likely represents two forms of KIR2DL1, differentially phosphorylated on ITIMs. The KIR2DL1*250 mutant migrates faster through the gel because of its smaller size and mass, and it is not phosphorylated. YTS cells were used as an additional negative control.
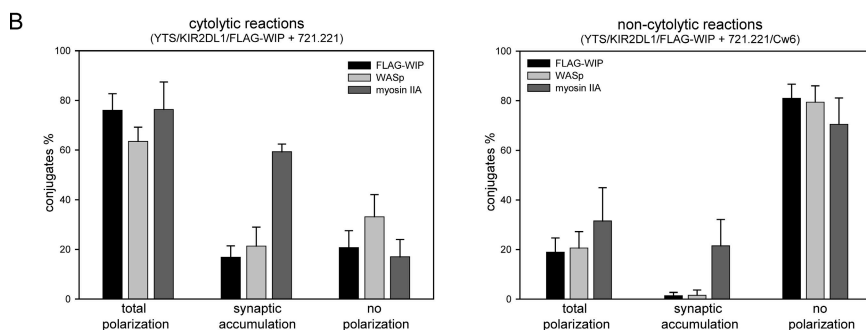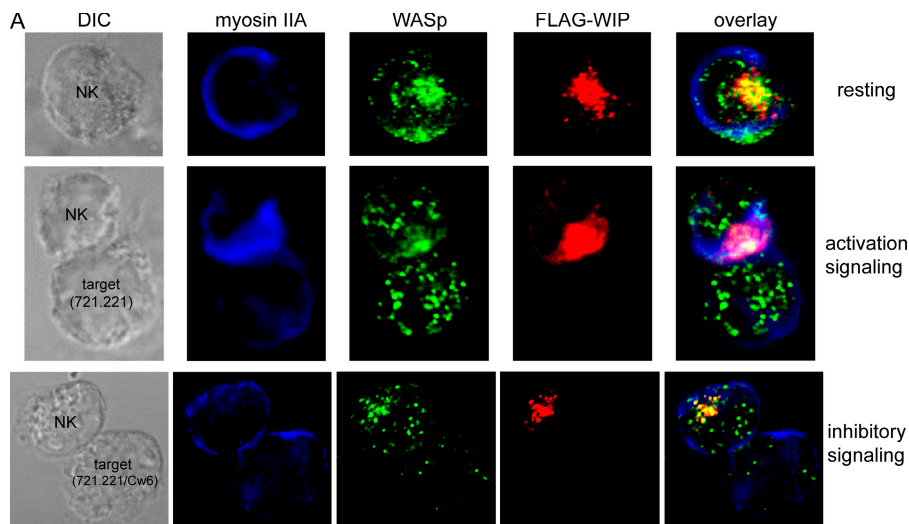
Figure 4. **WIP, WASp, and myosin IIA polarize to cell–cell contact site after NK cell activation, but not after inhibition.** YTS/KIR2DL1/FLAG-WIP cells, unconjugated or conjugated by mixing for 10 min at 37°C with target cells (either 721.221 [cytolytic interaction] or 721.221/Cw6 [noncytolytic interaction]), were stained with anti-WASp mAb followed by Alexa Fluor 647–conjugated goat anti–mouse (green), anti–myosin IIA followed by Alexa Fluor 405–conjugated goat anti–rabbit (blue), and Cy3-conjugated anti-FLAG mAb (red). NK cells were identified by GFP fluorescence and anti-FLAG staining. (A) Images of FLAG-WIP, WASp, and myosin IIA localization in resting cells (top), cytolytic (middle), and noncytolytic (bottom) conjugates. (B) The percentages of FLAG-WIP, WASp, and myosin IIA polarization toward cell–cell contact site in cytolytic (left) or noncytolytic (right) conjugates. Percentages of proteins polarized and found at the contact site (total polarization), or proteins found exclusively at the cell–cell contact site (synaptic accumulation), and percentages of proteins not polarized to the contact site (no polarization) $\pm$ SD are shown. The values were determined by evaluation of 150–200 conjugates in 3–4 separate experiments. $n = 200$ for WIP and WASp; $n = 150$ for myosin IIA.

of the WIP–WASp complex polarized to a region adjacent to the synapse. Myosin IIA accumulated at the contact site in 60% of conjugates (Fig. 4 B) and colocalized with FLAG-WIP (Fig. 4 A). F-actin also accumulated at the cell–cell interface in 85% of cytolytic conjugates (Fig. S1), in agreement with a previous study (Orange et al., 2003). Strikingly, in conjugates formed between NK cells and nonsusceptible 721.221/Cw6 cells (that initiated inhibitory signaling in YTS/KIR2DL1/FLAG-WIP cells) neither FLAG-WIP, WASp, myosin IIA, nor F-actin polarized toward the cell–cell contact site (Fig. 4 B and Fig. S1), and the distribution of those proteins remained as it was in resting cells (Fig. 4 A). Thus, in response to NK cell activation, WIP, WASp, and myosin IIA moved to the cell–cell contact site with F-actin, whereas KIR2DL1 inhibitory signaling prevented that relocalization.

### Recruitment of actin and myosin IIA is independent of WASp

The constitutive presence of a WIP–WASp complex, which is unaffected by inhibitory signaling (Fig. 1 C), raised the question of whether recruitment of actin and myosin IIA to the complex is mediated by WIP or by WASp. To test this, the YTS/KIR2DL1 cells were transfected with a WIP COOH-terminal deletion mutant, FLAG-WIPΔ460–503, which excluded the region required for WASp binding (amino acid residues 461–485 of WIP; Volkman et al., 2002) and should prevent WASp from binding to WIP. Analysis of anti-FLAG immunoprecipitates from activated YTS/KIR2DL1/FLAG-WIPΔ460–503

cells revealed that WASp was not pulled down by the mutant FLAG-WIPΔ460–503 protein. However, this mutant still allowed recruitment of actin and myosin IIA to the complex (Fig. 5). Recruitment of actin, but not myosin IIA heavy chain, to the complex was decreased in activated YTS/KIR2DL1/FLAG-WIPΔ460–503 cells, suggesting that WASp may contribute to actin recruitment or deletion of the COOH-terminal part of WIP and may cause changes in WIP conformation affecting actin binding. Nevertheless, actin and myosin IIA interaction with the complex appears to be independent of WASp and to require functional WIP.

### WIP phosphorylation is affected by NK cell signaling

To assess possible mechanisms governing the control of generation of the multiprotein complex, phosphorylation of the complex components was examined. Because WIP, but not WASp, seemed to play an essential role in complex formation (Fig. 5), WIP phosphorylation in response to NK cell signaling was investigated, with particular focus on whether the phosphorylation of WIP could affect interaction of the components of the complex with WIP.

WIP has been shown to undergo phosphorylation in T cells in response to CD3ζ chain cross-linking (Sasahara et al., 2002). Because WIP has been demonstrated to inhibit WASp activity in vitro (Martinez-Quiles et al., 2001), it has been proposed that WIP phosphorylation allows WASp to dissociate form WIP, releasing WASp from WIP inhibition (Sasahara et al., 2002).
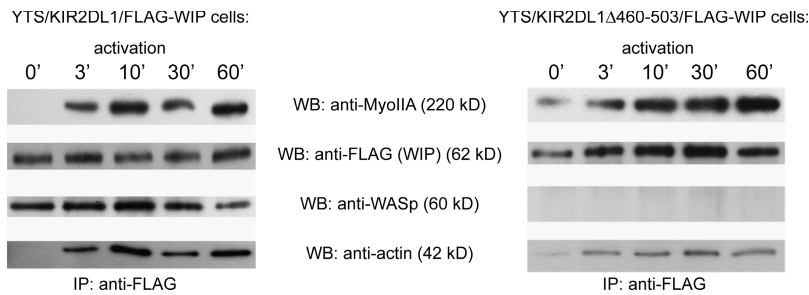
**YTS/KIR2DL1/FLAG-WIP cells:**

activation

0'  3'  10'  30'  60'

WB: anti-MyoIIA (220 kD)

WB: anti-FLAG (WIP) (62 kD)

WB: anti-WASp (60 kD)

WB: anti-actin (42 kD)

IP: anti-FLAG

**YTS/KIR2DL1Δ460-503/FLAG-WIP cells:**

activation

0'  3'  10'  30'  60'

IP: anti-FLAG

Figure 5. **Actin and myosin IIA interaction with WIP is independent of WASp.** YTS/KIR2DL1 cells were transfected with FLAG-WIP or FLAG-WIPΔ460-503, which is a mutant protein lacking the WASp binding domain. Transfected cells were mixed with 721.221 cells and incubated for the indicated times at 37°C. Cell lysates were immunoprecipitated with anti-FLAG mAb. Immunoprecipitated proteins were immunoblotted with anti–myosin IIA, anti-FLAG, anti-WASp, or anti-actin antibodies. The molecular masses of the proteins according to their position relative to molecular mass markers are shown in parentheses. The absence of WASp does not affect recruitment of either actin or myosin IIA to the complex.

To evaluate this paradigm in NK cells the YTS/KIR2DL1/FLAG-WIP cells were labeled with [$^{32}$P]orthophosphate and WIP phosphorylation status was analyzed after the activation and inhibition of NK cells with appropriate target cells.

After the activation or inhibition of NK cells, anti-FLAG immunoprecipitation demonstrated two distinct bands of phosphoproteins, which were identified as WIP and WASp by immunoblotting (Fig. 6 A). WIP was phosphorylated even at time 0, suggesting that WIP is constitutively phosphorylated in NK cells. WIP phosphorylation quickly increased upon NK cell activation to twofold after 3 min, reached a maximum of 2.7-fold at 10 min, and decreased to the basal level at 60 min (Fig. 6 B). On the contrary, inhibition of NK cell activity by KIR2DL1 signaling resulted in no increase of WIP phosphorylation, indicating that KIR2DL1 inhibitory signaling influenced WIP phosphorylation. Surprisingly, phosphorylation of WASp pulled down by FLAG-WIP did not change significantly during activation or inhibition of NK cells (unpublished data), but it is likely that small WASp phosphorylation changes could have been undetected because of its constitutive phosphorylation at S483 and S484 (Cory et al., 2003). Most interestingly, changes in WIP phosphorylation states did not affect WASp binding, as defined by coimmunoprecipitation studies, indicating that the function of WIP phosphorylation during NK cell activation may be different than the regulation of WIP–WASp association.

Thus, the WIP phosphorylation level changes in response to NK cell signaling. WIP may be phosphorylated at several different sites, as judged by the increase of WIP phosphorylation, in addition to the basal level of phosphorylation. Increase and inhibition of WIP phosphorylation by activation and inhibitory signaling, respectively, suggests that WIP phosphorylation may be important for proper WIP function.

### WIP phosphorylation in NK cells is mediated by PKCθ

WIP was indirectly shown to be phosphorylated by PKCθ in T cells (Sasahara et al., 2002). Because WIP phosphorylation was not required for regulation of the WIP–WASp interaction, the possible function of WIP phosphorylation and the identification of the kinase responsible for WIP phosphorylation in NK cells were examined next.

Analysis of the WIP amino acid sequence revealed several consensus sites for PKC- and casein kinase II–mediated phosphorylation. Consequently, the effects of PKC and CKII inhibitors were studied. To test if PKC family kinases are involved, a general inhibitor of PKC activity, bisindolylmaleimide I, was used, as well as PKCα,β and PKCθ pseudosubstrates, which specifically inhibit activity of α, β, and θ isoenzymes, respectively. Pretreatment with bisindolylmaleimide I, followed by activation of YTS/KIR2DL1/FLAG-WIP cells, resulted in severely decreased WIP phosphorylation compared with control, indicating that a PKC family kinase is involved in WIP phosphorylation (Fig. 6 C). Pretreatment of cells with PKCα,β pseudosubstrate (Fig. 6 C) or with Gö6983, which inhibits PKC α, β, γ, δ, and ζ (80 nM; unpublished data), did not decrease WIP phosphorylation after NK cell activation, suggesting that these isoenzymes were not involved in WIP phosphorylation. Similarly, treatment with specific CKII inhibitor did not affect the WIP phosphorylation state, indicating that casein kinase II is not involved in WIP phosphorylation in vivo. However, pretreatment with the pseudosubstrate inhibitor specific for PKCθ prevented WIP phosphorylation after NK cell stimulation (Fig. 6 C), demonstrating that PKCθ is responsible for phosphorylation of WIP in NK cells.

### WIP phosphorylation is correlated with multiprotein complex formation

Because WIP phosphorylation was affected by activation and inhibitory signaling (Fig. 6), and the changes in WIP phosphorylation levels over time appeared to be related to recruitment of actin and myosin IIA to the complex, the relationship between the WIP phosphorylation state and the formation of the multiprotein complex was investigated.

In addition to PKC inhibitors, the YTS/KIR2DL1/FLAG-WIP cells were treated with protein tyrosine kinase inhibitors. Myosin inhibitors were also used to assess the effect of myosin activity inhibition on complex formation. Pretreatment of cells before activation with an Src family kinase inhibitor, PP2, did not affect WIP phosphorylation or the recruitment of actin and myosin IIA to the complex, indicating that complex formation is not regulated by Src family kinases (Fig. 6 C). A decrease of actin and myosin IIA binding was seen after treatment with 100 μM of the general protein tyrosine kinase inhibitor genistein
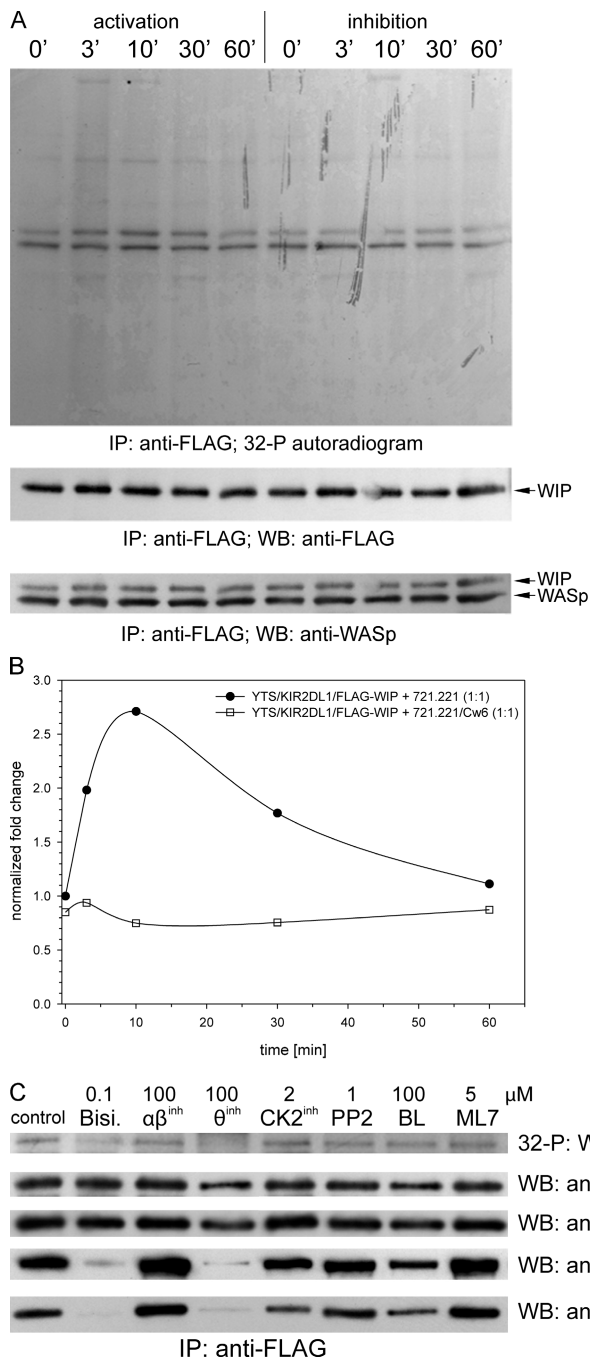
**Figure 6. WIP phosphorylation increases upon NK cell activation and is mediated by PKCθ.** (A) $^{32}$P-labeled YTS/KIR2DL1/FLAG-WIP cells were mixed with target cells, either 721.221 or 721.221/Cw6, transferred to 37°C, and incubated for the indicated times. Cell lysates were immunoprecipitated with anti-FLAG mAb. Immunoprecipitated proteins were visualized by autoradiography and then immunoblotted with anti-FLAG antibody, followed by anti-WASp to visualize WIP and WASp, respectively. (B) Quantitation of WIP phosphorylation showing increase of WIP phosphorylation with maximum at 10 min of NK cell activation. The increase in phosphorylation of WIP was assessed by densitometry, which was normalized to phosphorylation observed at time 0 min and expressed as arbitrary units. (C) $^{32}$P-labeled cells were pretreated with 0.1% DMSO (control) or with the following kinase inhibitors: bisindolylmaleimide I (Bisi.), PKCα and PKCβ pseudosubstrate inhibitor (αβ$^{inh}$), PKCθ pseudosubstrate inhibitor (θ$^{inh}$), casein kinase II inhibitor (CK2$^{inh}$), Src family kinase inhibitor (PP2), blebbistatin (BL), or ML-7 at the indicated concentrations for 30 min at 37°C, followed by mixing with 721.221 cells for 10 min at 37°C. Cell lysates were immunoprecipitated with anti-FLAG mAb and treated similar to the cells in A, but using anti-FLAG, anti–myosin IIA, anti-actin, and anti-WASp antibodies sequentially for immunoblotting. The molecular masses of the proteins according to their position relative to molecular mass markers are shown in parentheses.
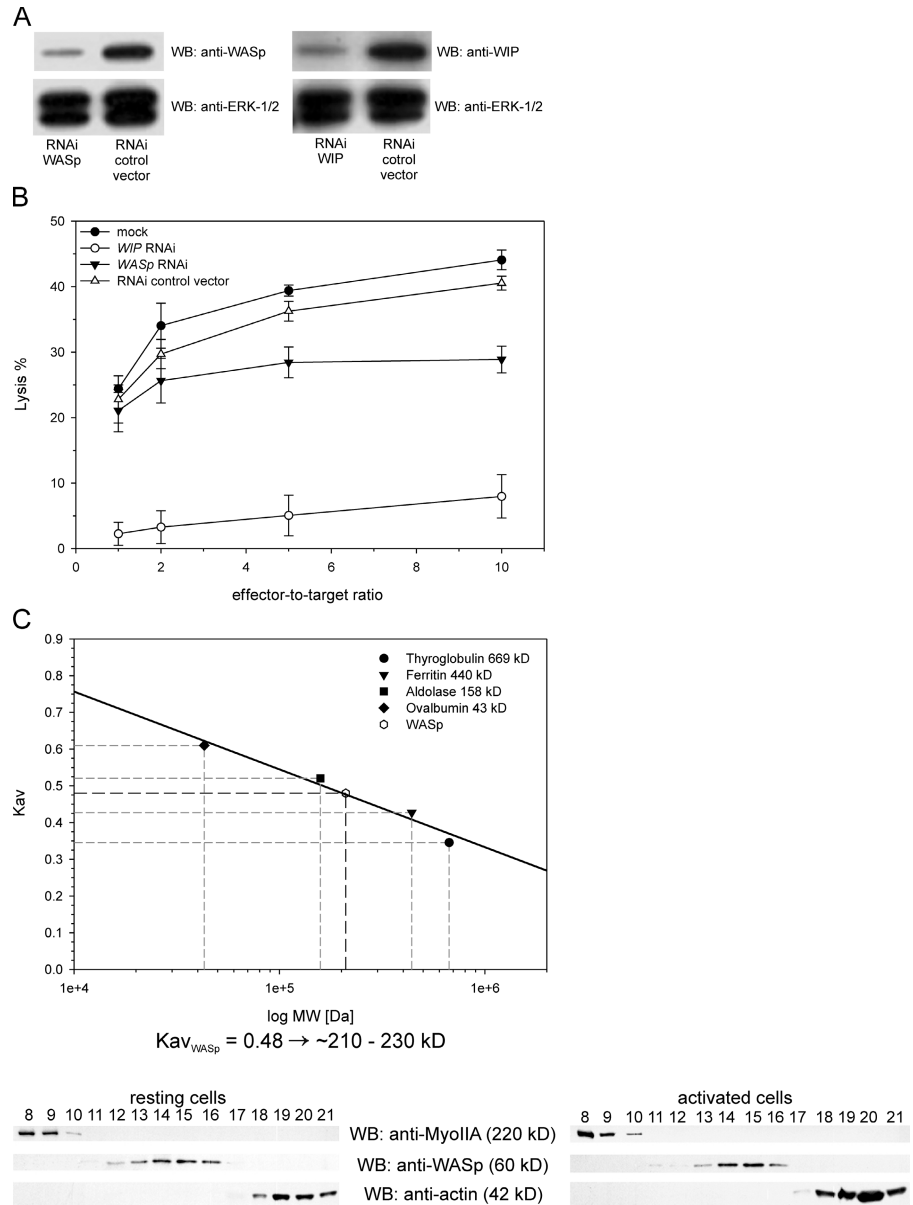
(unpublished data), but the observed effects were most likely nonspecific because of the inhibition of all tyrosine kinases and an impairment of NK cell functions. Inhibition of myosin IIA heavy chain or regulatory light chain activity with blebbistatin or ML-7, respectively, before NK cell activation did not disrupt complex formation. However, inhibition of PKC activity abolished both actin and myosin IIA, but not WASp, recruitment. The disruption of complex formation was caused only by specific inhibition of the θ and not the α, β (Fig. 6 C), γ, δ, or ζ (unpublished data) isoforms. Hence, PKCθ activity is crucial for WIP phosphorylation. The results suggest that actin and myosin IIA recruitment and proper complex formation during NK cell activation correlate with WIP phosphorylation and are likely dependent on it.

Chemical inhibitors of PKC activity blocked NK cell cytotoxicity (unpublished data). However, the analysis of the effects of PKC inhibitors in regard to the multiprotein complex involvement in NK cell cytotoxicity is extremely complicated because the long-term effects of PKC inhibitors, as opposed to the short-term effects, are very broad, influencing many intracellular pathways.

### Effects of *WIP* and *WASp* RNAi

Changes in WIP phosphorylation during NK cell activation that correlate with complex formation, combined with the observation that the WIP–WASp interaction is independent of the WIP phosphorylation state (Fig. 6), implicate WIP in functions

other than WASp regulation. To assess WIP functionality in NK cells, RNAi was used to decrease WIP expression in NK cells (Fig. 7). Introduction of *WIP* siRNA to YTS/KIR2DL1 cells caused a marked reduction of WIP expression and almost complete inhibition of NK cell cytotoxicity (Fig. 7, A and B). Comparatively, *WASp* RNAi in YTS/KIR2DL1 cells resulted in only a moderate decrease in cytotoxicity. Importantly, *WIP* RNAi eliminated the formation of the multiprotein complex. Gel filtration analysis of WASp, actin, and myosin IIA from *WIP* RNAi-treated cells revealed that these proteins were eluted with the same partition coefficients in both resting and activated cells, indicating that the complex was not assembled in these cells (Fig. 7 C).

Thus, WIP has a vital role in multiprotein complex formation and is indispensable for NK cell cytotoxic activity. Moreover, controlled assembly of the multiprotein complex as the result of NK cell signaling indicates the importance of the complex in NK cell cytolytic activity.

## Discussion

Actin rearrangements are vital for cell conjugation and immune synapse formation in NK and T cells. Formation of filamentous actin rings at the immune synapse allows for firm coupling of the lymphocyte with its target cell and ensures localized delivery of lytic granules (Stinchcombe et al., 2001; Vyas et al., 2002). Cytoskeleton reorganization is required for sustained signaling in T cells (Valensin et al., 2002), and inhibition of actin polymerization by cytochalasin D prevents NK cell cytotoxic activity (Orange et al., 2003). Thus, affecting the actin cytoskeleton itself or key regulatory proteins involved in its polymerization may present a rapid mechanism for inhibiting cytotoxic activity.

We show that activation of NK cells by their target cells results in formation of a large (~1.3 mD) multiprotein complex comprised of WIP, WASp, actin, and myosin IIA. These proteins polarize to the cell–cell contact site after NK cell activation

(Fig. 4 and Fig. S1). Assembly and localization of this complex is affected by KIR inhibitory signaling (Fig. 1, A and C; and Fig. 4), indicating the functional significance of the complex for NK cell activity. The essential role of WIP in the formation of this complex and in cytotoxicity may be dependent on its phosphorylation by PKCθ (Figs. 5–7). WIP, which was originally identified as a protein interacting with WASp (Ramesh et al., 1997), was later shown to bind both monomeric and filamentous actin, to retard WASp-mediated actin polymerization in vitro (Martinez-Quiles et al., 2001), and to have a variety of functions, many related to cell signaling and motility (Anton et al., 2002, 2003; Sasahara et al., 2002; Kinley et al., 2003; Kettner et al., 2004).

WIP immunoprecipitates from activated YTS cells contain WASp, actin, and myosin IIA (Fig. 1, A and C; and Table I). The presence of actin and WASp in WIP preparations is in agreement with previous studies (Ramesh et al., 1997; Martinez-Quiles et al., 2001). Actin association with WIP is independent of WASp, as indicated by the fact that a WIP mutant unable to interact with WASp interacts with actin (Fig. 5). However, this association is dependent on WIP, as the disruption of WIP expression inhibits multiprotein complex formation (Fig. 7 C). Actin binds to the NH$_2$-terminal part of WIP (Martinez-Quiles et al., 2001), whereas WASp interacts with the WIP COOH-terminal sequences (Ramesh et al., 1997; Anton et al., 1998), explaining the observed result. However, the decreased amount of actin in WIPΔ460–503 immunoprecipitates suggests that WASp may contribute to actin recruitment, likely by binding additional actin monomers (Miki and Takenawa, 1998).

The presence of a class II myosin as the part of the multiprotein complex (Fig. 1 and Table I) has not been described previously. A WIP homologue from yeast, verprolin, has been demonstrated to bind class I myosin (Anderson et al., 1998; Geli et al., 2000). Although mammalian class I myosin function is still poorly understood, recent studies provide support for the role of another member of the myosin family, myosin IIA, in leukocyte activity (Swanson et al., 1999; Rey et al., 2002; Jacobelli et al., 2004; Bastian et al., 2005). WIP, as well as WIPΔ460–503, immunoprecipitates from YTS cells contain myosin IIA, indicating an interaction between WIP and myosin IIA. This interaction could be direct, through actin, or through some other protein.

WIP and WASp from nonstimulated cells segregate as an ∼350–400 kD protein complex. Purified WASp and its homologue N-WASp were previously shown to behave as dimers or multimers (Carlier et al., 2000; Higgs and Pollard, 2000). WASp dimer, together with bound WIP, would create a heterotetramer structure with a theoretical mass of ∼250–260 kD. Additionally, WASp is known to bind a plethora of adaptor and regulatory proteins (Orange et al., 2004), which could explain why WIP and WASp are parts of an ∼400 kD complex. Importantly, neither myosin IIA nor actin is observed in the fractions containing WIP and WASp, demonstrating that the complex is not formed in nonactivated NK cells (Fig. 2 A). Activation of YTS cells results in the shift of a substantial portion of WIP and WASp to the high molecular mass fraction, accompanied by the occurrence of myosin IIA and actin in the same fraction

(Fig. 2 B), indicating that the multiprotein complex is formed in response to NK cell stimulation. The main protein contributing to the mass of ∼1.3 mD is myosin II because class II myosins are composed of two heavy (171–244 kD) and four light (16–25 kD) chains, forming a heterohexamer (Sellers, 2000).

Activation of YTS cells by target cells causes association of actin and myosin IIA with WIP and WASp. The complex is formed after 3 min of activation, with the maximum actin and myosin IIA recruitment at 10 min (Fig. 1). The short time that the myosin IIA regulatory chain is present suggests that myosin IIA is fully functional only for the short period required to fulfill its task. However, the prolonged association of myosin IIA heavy chain with the complex (Fig. 1) may favor a quick response to new stimuli, as short-term interaction would generate a delay required for full myosin IIA heterohexamer assembly. Because the role of myosin IIA in the complex is not clear yet, one speculation would be that myosin IIA moves along the actin cytoskeleton and transports WIP and WASp as "cargo" to places of dynamic actin assembly, i.e., the immune synapse. F-actin is a part of the complex (Fig. 1 E). In cytolytic conjugates, WIP and WASp polarize to the cell–cell contact site where myosin IIA and F-actin also accumulate (Fig. 4 and Fig. S1). After translocation to the cell–cell interface, WASp could stimulate the Arp2/3 complex to create new actin branches (Higgs and Pollard, 2000), whereas WIP could stabilize newly formed actin filaments (Martinez-Quiles et al., 2001) to allow more firm cell–cell coupling and provide a rigid scaffold for the immune synapse.

Signaling-deficient KIR2DL1*250 provides support for a specific role of KIR2DL1 in the inhibition of multiprotein complex formation. Contrary to wild-type KIR2DL1, recognition of HLA-Cw6 on the surface of target cell by KIR2DL1*250 has no effect on complex formation, indicating that the complex is formed despite the recognition of MHC class I ligand (Fig. 1, C and D). Thus, KIR2DL1-mediated signaling can affect proteins involved in actin cytoskeleton reorganization. Inhibitory signaling may affect the complex directly by dephosphorylating one or more of its components, or indirectly by targeting regulatory molecules (e.g., adaptors and kinases) upstream of WIP and WASp. Phosphorylation of KIR ITIMs results in the recruitment of SHP-1 and -2 phosphatases (Olcese et al., 1996), which may dephosphorylate Vav-1, thus preventing Cdc42 activation. At the least, a "trapping" mutant of SHP-1 binds Vav-1 (Stebbins et al., 2003). Because Cdc42 is required for WASp activation and myosin IIA activity (Vicente-Manzanares and Sanchez-Madrid, 2004), Vav-1 dephosphorylation by SHP-1 could affect complex functionality. The possibility that SHP-1 and -2 dephosphorylate several of the regulatory components, as well as proteins of the multiprotein complex, needs to be carefully examined.

A specific inhibitor for PKCθ effectively prevents WIP phosphorylation, showing that WIP is phosphorylated by PKCθ in NK cells (Fig. 6 C). This result, directly demonstrating WIP phosphorylation in vivo, supports previous indications of PKCθ involvement in WIP phosphorylation in T cells using WIP-specific peptide antibodies (Sasahara et al., 2002). Interestingly, a low level of WIP phosphorylation was observed before NK cell stimulation (Fig. 6). WIP could be constitutively phosphorylated

at one site and undergo regulated phosphorylation at another site in response to cell stimulation. Ser488 of WIP was identified as a WIP phosphorylation site using mutagenesis (Sasahara et al., 2002), and this residue may be a site of regulated WIP phosphorylation. Further direct identification of WIP phosphorylation sites is an important next step.

Interestingly, no correlation between WIP phosphorylation level and WASp association is observed. WASp is associated with WIP even in resting cells (Figs. 2 and 4; unpublished data), suggesting constitutive interaction between WIP and WASp (in agreement with Ho et al. [2004]). WIP phosphorylation has been proposed to dissociate the WIP–WASp interaction in T cells (Sasahara et al., 2002). However, an increase of WIP phosphorylation in response to NK cell activation is not accompanied by a change in the amount of WASp bound to WIP. Inhibition of WIP phosphorylation also does not affect the WIP–WASp interaction (Fig. 6). Although WIP phosphorylation does not regulate interaction of WIP and WASp in NK cells, the inhibition of PKCθ activity, but not Src family kinases, and the consequent abrogation of WIP phosphorylation, prevents the recruitment of actin and myosin IIA to the complex. Additionally, inhibition of myosin II activity by blebbistatin and ML-7 does not influence interaction between any of the four proteins, indicating that myosin IIA activity is not required for the assembly (Fig. 6 C). Ligation of KIR2DL1 by HLA-Cw6 results in the suppression of WIP phosphorylation and correlates with prevention of a multiprotein complex formation (Fig. 6 B and Fig. 1 C).

Disruption of WIP expression by RNAi leads to a nearly complete loss of cytotoxicity, supporting the unique role of WIP in NK cell activity. In contrast, *WASp* RNAi results in incomplete loss of cytotoxicity (Fig. 7), similar to that observed in WAS patients (Orange et al., 2002). Because actin cytoskeleton rearrangements are essential for lymphocyte activation (Miletic et al., 2003), the observed defects in NK cell cytotoxic activity are, at least in part, likely caused by faulty actin polymerization. Alternatively, other proteins may be able to substitute for WASp function in activation of the Arp2/3 complex (Falet et al., 2002; Weaver et al., 2002). Depletion of WIP causes more profound effects. WIP regulates WASp activity (Martinez-Quiles et al., 2001), is required for cortical actin network organization (Anton et al., 2002), is essential for multiprotein complex formation, and appears to play a central role in NK cell cytotoxicity.

## Materials and methods

### Antibodies
Polyclonal anti-KIR2DL1 antibodies were generated using full-length KIR2DL1 protein. Other antibodies used include the following: anti-KIR mAb (EB6; Beckman Coulter), anti-WASp (US Biological), anti-WIP (a gift from R. Geha and N. Ramesh, Children's Hospital Boston, Boston, MA), anti-ERK1/2 (Upstate Biotechnology) and MOPC-21 (isotype control), anti-phosphotyrosine (PT66), anti–myosin IIA heavy chain, anti-FLAG (M2), anti-myosin regulatory light chain (MY-21), anti-actin, anti–rabbit IgG (RG-96), and anti–mouse IgG (all from Sigma-Aldrich). Alexa Fluor–conjugated reagents were obtained from Invitrogen.

### Plasmids and construct generation
The FLAG-pMX vector was generated using pMX-IRES-GFP plasmid (a gift from T. Kitamura, University of Tokyo, Tokyo, Japan). Two DNA oligos encoding the FLAG peptide sequence were inserted into pMX-IRES-GFP

vector to generate FLAG-pMX-IRES-GFP plasmid. The full-length WIP and WIPΔ460–503 cDNA were amplified from pcDNA3-WIP vector (a gift from N. Ramesh). Both, WIP and WIPΔ460–503 constructs were subsequently inserted into the FLAG-pMX-IRES-GFP vector. All constructs were verified by DNA sequencing analysis.

### WIP and WASp RNAi
Vector-based RNAi, targeting the GGAGGTTTCCTGTGCCTTCT sequence of WIP and the GGGAACAGGAGCTGTACTCAC sequence of WASp, was used to generate WIP and WASp knockdown cell lines. Two DNA oligos designed to produce WIP or WASp siRNA hairpins were inserted into pBS-U6-RNAi-dual-CMV-GFP vector (a gift from X. Liu, Harvard University, Cambridge, MA; Sui et al., 2002; Tang et al., 2004). The construct was verified by DNA sequencing and introduced into YTS/KIR2DL1 cells using Nucleofector I (Amaxa Biosystems). The GFP-positive cells were sorted using a MoFlo high performance cell sorter (DakoCytomation). WIP or WASp expression levels in each stable cell clone were examined by Western Blot. The clones with a substantial decrease of WIP or WASp expression levels were selected and sorted. At least six clones were analyzed and one representative clone was used for studies of WIP or WASp knockdown effects.

### Cells, WIP, and WASp transfectants
Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, and 1.6 mg/ml genectin (Invitrogen) or 2 μg/ml puromycin (Sigma-Aldrich). FLAG-WIP and FLAG-WIPΔ460–503 constructs were transfected into the retrovirus-packaging Plat-E 293 Eco cell line (Morita et al., 2000). Retroviruses were used to infect tumor NK YTS/KIR2DL1 or YTS/KIR2DL1*250 cell lines, as previously described (Cohen et al., 1999). GFP-positive transfected cells were sorted using a MoFlo high performance cell sorter.

### Cell stimulation, Western blotting, immunoprecipitation, and silver staining
For antibody-mediated KIR2DL1 cross-linking, $4 \times 10^6$ YTS/KIR2DL1 cells were incubated with EB6-coated protein G magnetic beads (2.8 μm; Dynal) for 3, 10, or 30 min at 37°C in PBS supplemented with 2% FCS. For negative controls, $4 \times 10^6$ YTS/KIR2DL1 cells were incubated with MOPC21-coated protein G magnetic beads and $4 \times 10^6$ YTS or YTS/KIR2DL1*250 cells were incubated with EB6-coated protein G magnetic beads for 3 min at 37°C in PBS/2% FCS. After stimulation, cells were mixed with an equal volume of ice-cold 2× lysis buffer and lysed for 30 min at 4°C. Magnetic beads were isolated from the total cell lysates using a magnet (Dynal) and washed extensively with ice-cold lysis buffer (1% NP-40, 50 mM TRIS/HCl, pH 7.4, 150 mM NaCl, and 1 mM Na₃VO₄).

For cell mixing, $2 \times 10^6$ cells, either YTS/KIR2DL1/FLAG-WIP, YTS/KIR2DL1-FLAG-WIPΔ460–503, or YTS/KIR2DL1*250/FLAG-WIP, were mixed with $2 \times 10^6$ 721.221 or 721.221/Cw6 cells in complete RPMI medium. Mixed cells were immediately transferred to a 37°C water bath and incubated for 3, 10, 30, or 60 min, followed by brief centrifugation and lysis in ice-cold lysis buffer for 30 min at 4°C. For time 0, cells were mixed and immediately lysed. Cleared cell lysates were incubated with anti-FLAG mAb coupled to protein G Sepharose beads (Sigma-Aldrich). Sepharose beads were pelleted by centrifugation (5,000 RPM for 2 min at 4°C) and washed extensively with ice-cold lysis buffer.

In experiments involving anti-actin drugs, the following compounds were used: 20 and 50 μM latrunculin A or B, 1 μM jasplakinolide A, and 0.5 μM swinholide A. YTS/KIR2DL1/FLAG-WIP cells were pretreated with anti-actin drugs or 0.1% DMSO (controls) for 30 min at 37°C, followed by mixing with 721.221 target cells for 10 min at 37°C. Cell lysates were immunoprecipitated with anti-FLAG antibody.

Immunoprecipitated proteins were resolved on 4–12% NuPage gels (Invitrogen) and either stained with a Silver Staining kit (GE Healthcare) or transferred to a PVDF membrane (Invitrogen). The membrane was blocked with 1% BSA in TBS with Tween-20, followed by incubation with a primary antibody. The membrane was then incubated with HRP-conjugated secondary antibodies, either anti–rabbit or anti–mouse, unless HRP-conjugated primary antibodies were used. Immunoblots were developed using ECL Western Blotting detection reagents (GE Healthcare).

### [³²P]orthophosphate labeling and kinase inhibition
$2 \times 10^6$ YTS/KIR2DL1/FLAG-WIP cells were labeled for 4 h at 37°C with [³²P]orthophosphate (0.2 mCi/ml, 285.6 Ci/mg; Perkin Elmer) in phosphate-free RPMI 1640 media (CHEMICON International, Inc.) supplemented with 10% phosphate-free FCS. Labeled cells were mixed with either 721.221 or 721.221/Cw6 target cells and incubated for 3, 10, 30, or 60 min at 37°C,

lysed, and immunoprecipitated with anti-FLAG antibody. Radiolabeled proteins were visualized by autoradiography. Incorporated radioactivity was quantified using SigmaGel software v1.0 (Jandel Scientific), normalized to phosphorylation, observed at time 0, and expressed as arbitrary units. In experiments involving kinase inhibitors, the following inhibitors were used: 100 nM bisindolylmaleimide I, 100 μM PKCα,β pseudosubstrate inhibitor, 100 μM PKCθ pseudosubstrate inhibitor, 2 μM casein kinase II inhibitor, 1 μM PP2, 100 μM blebbistatin, and 5 μM ML-7 (all from Calbiochem). Labeled cells were treated with kinase inhibitors or 0.1% DMSO (control) for 30 min at 37°C, followed by mixing with 721.221 target cells for 10 min at 37°C, cell lysis, and immunoprecipitation with anti-FLAG antibody. Immunoprecipitated proteins were visualized by autoradiography and subsequently blotted with the indicated antibodies.

### [51]Cr release assay
YTS/KIR2DL1 cell cytotoxicity was evaluated by ^{51}Cr release assay as previously described (Orange et al., 2002).

### Gel filtration
Gel filtration was performed on a Superose 6 column (106.5 ml; GE Healthcare) using a BioLogic Workstation (Bio-Rad Laboratories). Cleared cell lysates from $2 \times 10^7$ YTS/KIR2DL1/FLAG-WIP or YTS/KIR2DL1/dsRNA*WIP* cells, either resting or stimulated with 721.221 (1:1 ratio), were applied to the Superose 6 column. Samples were eluted at 4°C with TBS and 1.25-ml fractions were collected. Proteins from each fraction were precipitated with 10% TCA and the neighboring fractions (e.g., 1 + 2, 3 + 4, etc.) were pooled to reduce the number of samples for analysis. Precipitated proteins, which were transferred to PVDF membrane, were immunoblotted with anti–myosin IIA, anti-FLAG, anti-WASp, or anti-actin antibodies. Calibration curves were prepared by measuring the partition coefficients ($K_{av}$) of molecular mass markers (GE Healthcare) and plotting them against log molecular mass. $K_{av}$ values for proteins of interest were calculated based on elution volumes and were fitted to the calibration curve. All partition coefficients were calculated using the following equation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o},$$

where $V_e$ = elution volume for the protein, $V_o$ = void volume of column, and $V_t$ = total volume of column.

### Protein identification by MS
YTS/KIR2DL1/FLAG-WIP cells were stimulated, lysed, and cleared, and cell lysates were immunoprecipitated with anti-FLAG mAbs and resolved on a 4–12% NuPage gel. Gels were stained with a Colloidal blue staining kit (Invitrogen), and individual protein bands were subsequently excised and digested with 12.5 ng/μl trypsin (Promega). Resultant protein samples were analyzed using a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Corp). Ion trap spectra were obtained and MS data was analyzed using the Sequest Cluster with BioWorks software, which was supplied by the manufacturer. Certain peptides were further positively identified by MS peptide sequencing, which was facilitated through the use of the ProFound peptide mapping software (The Rockefeller University). MS experiments and analysis were performed in the proteomics facility of the Children's Hospital of Philadelphia.

### Cell conjugation and microscopy
YTS/KIR2DL1/FLAG-WIP cells were conjugated to either 721.221 or 721.221/Cw6 cells at a 1:1 ratio for 10 min at 37°C in complete RPMI medium, followed by adherence to poly-L-lysine–coated slides (Sigma-Aldrich) for 15 min at 37°C. Next, the adherent cells were washed with PBS, fixed, and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) supplemented with 0.1% Triton X-100. After blocking with 1% BSA, cells were stained with anti-WASp mAbs followed by Alexa Fluor 647–conjugated goat anti–mouse, anti–myosin IIA followed by Alexa Fluor 405–conjugated goat anti–rabbit, and Cy3-cojnugated anti-FLAG mAb. Antibodies were used in the range of 1–20 μg/ml. F-actin was stained using Alexa Fluor 647–conjugated phalloidin. Cell conjugates were visualized by a laser-scanning confocal microscope (LSM510 Axiovert 100M; Carl Zeiss MicroImaging, Inc.). NK cells were identified by GFP fluorescence and positive anti-FLAG staining. The percentage values were determined by evaluation of 150–200 conjugates in randomly selected fields, in 3–4 separate experiments. The images were obtained using 63× Plan-Apochromat objective and LSM510 software v. 3.2 (both Carl Zeiss MicroImaging, Inc.). Because of extensive photobleaching, the γ value of blue channel was increased from 1.0 to 1.4.

### Online supplemental material
Fig. S1 shows the localization of F-actin and FLAG-WIP in YTS/KIR2DL1/FLAG-WIP cells after the formation of cytolytic and noncytolytic conjugates with target cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200509076/DC1.

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