WASP-Interacting Protein (WIPa) is Important for Actin Filament Elongation and Prompt Pseudopod Formation in Response to a Dynamic Chemoattractant Gradient

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ABSTRACT

The role of WASP-interacting protein (WIP) in the process of F-actin assembly during chemotaxis of *Dictyostelium* was examined. Mutations of the WH1 domain of WASP led to a reduction in binding to WIPa, a newly identified homolog of mammalian WIP, a reduction of F-actin polymerization at the leading edge, and a reduction in chemotactic efficiency. WIPa localizes to sites of new pseudopod protrusion and colocalizes with WASP at the leading edge. WIPa increases F-actin elongation *in vivo* and *in vitro* in a WASP-dependent manner. WIPa translocates to the cortical membrane upon uniform cAMP stimulation in a time-course that parallels F-actin polymerization. WIPa overexpressing cells exhibit multiple microspike formation and defects in chemotactic efficiency due to frequent changes of direction. Reduced expression of WIPa by expressing a hairpin WIPa (hp WIPa) construct resulted in more polarized cells that exhibit a delayed response to a new chemoattractant source due to delayed extension of pseudopod toward the new gradient. These results suggest that WIPa is required for new pseudopod protrusion and prompt reorientation of cells toward a new gradient by initiating localized bursts of actin polymerization and/or elongation.
INTRODUCTION

Many eukaryotic cells undergo chemotaxis, directed movement toward a soluble ligand. This process is necessary for many biological functions, including the migration of macrophage during wound healing in vertebrates (Devreotes P.N., 1988; Martinez-Quiles, 2001), axonal outgrowth to target cells (Korey, 2000), and the aggregation of *Dictyostelium* cells to form a multicellular organism (Chung, 2001; Devreotes, 1999). Chemotaxis also plays a role in disease states such as arthritis, cancer, and multiple sclerosis (Bagiolini, 2001). Directional cell movement requires a defined cell polarity in which components of the cytoskeleton are differentially localized at the leading edge of a migrating cell as well as its retracting posterior (Chung, 2001; Firtel, 2000). This polarization can be initiated by the chemoattractant binding of heterotrimeric G protein-coupled membrane-bound receptors, and subsequent activation of downstream signal transduction pathways direct reorganization of the actin and myosin cytoskeleton (Chung, 2001; Van Haastert, 2004). A significant focus of current research involves the elucidation of the pathways that mediate both cell polarization and directional sensing, the result of which makes up a cell’s proper chemotactic response.

Wiskott-Aldrich Syndrome (WAS) is a human X-linked immunodeficiency characterized by recurrent infections, hematopoietic malignancies, eczema, and thrombocytopenia (Aldrich, 1954; Derry, 1994; Snapper, 1998). These defects are caused by mutations in the gene encoding the Wiskott-Aldrich Syndrome Protein (WASP), a key adaptor protein that connects multiple signaling pathways to F-actin polymerization, a process essential for chemotaxis. The WASP family of proteins includes WASP, N-WASP, and SCAR or WAVE (Bear, 1998; Miki, 1996). We have previously confirmed the necessity of WASP during the chemotactic motility of *Dictyostelium* via targeted knockdown of this gene (Myers, 2005). Lowered expression of WASP significantly impaired the ability of cells to polarize, polymerize F-actin, and migrate toward the chemoattractant cAMP. Both WASP and N-WASP possess a WH1 (WASP homology 1) domain, a basic (B) domain, a Cdc42/Rac binding (GBD) domain, a polyproline (SH3-binding) domain, a G-actin binding WH2 (WASP homology 2) domain (N-WASP has two WH2 domains), a central domain (C) and a C-terminal acidic (A)
segment (Symons, 1996; Zigmond, 2000). The importance of the WH1 domain has been suggested by the frequency of mutations found to cause WAS (Rong, 2000). Despite comprising only one-fifth of WASP, more than half of the known missense mutations causing WAS are present in the WH1 domain. The WH1 domain has been implicated in the recruitment of WASP family members to sites of actin polymerization during motility of vaccinia virus (Moreau, 2000), WIP-induced membrane protrusions (Vetterkind, 2002), and PI(4,5)P2-induced intracellular vesicles (Benesch, 2002).

The WASP interacting protein (WIP) has been shown to interact with human N-WASP, a ubiquitously expressed homolog, at the WH1 domain (Volkman, 2002). WIP is known to bind to both G- and F-actin (Martinez-Quiles, 2001). Depending on the system examined, WIP and other verprolin family members have been found to stimulate F-actin polymerization (Kinley, 2003; Ramesh, 1997), initiate filopodia formation (Martinez-Quiles, 2001; Vetterkind, 2002), and prevent F-actin depolymerization (Kato, 2002; Martinez-Quiles, 2001). WICH, a protein related to WIP, has been shown to induce microspike formation through cooperation with N-WASP (Kato, 2002). We have identified a homolog of mammalian WIP, WIPa, in Dictyostelium and examined the roles of WASP and WIP in the regulation of F-actin assembly and chemotaxis. The WH1 domain of WASP was found to bind WIPa. Overexpression of WIPa leads to an increase in microspike formation that is dependent upon its WH2 domain, WASP, and VASP. WIPa translocates to the cortex of cells uniformly stimulated with cAMP in a time-course that parallels F-actin polymerization. Lower levels of WIPa expression reduced prompt chemotactic responses of cells to a changing chemoattractant gradient. We conclude that WIPa is important for proper regulation of actin polymerization at sites of new pseudopod formation, which allows a chemotaxing cell the flexibility to respond to a spatially dynamic chemotactic gradient.
RESULTS

WH1 mutations lead to aberrant regulation of actin polymerization and chemotaxis

More than one-half of the known missense mutations causing WAS are found in the WH1 domain (Fig. 1A). Though many of these mutations lead to lowered protein expression (Snapper, 1999), some have been shown to affect WASP-WIP interaction (Stewart, 1999). A previous study identified an aromatic triad of residues within the EVH1 domain of Mena necessary for its interaction with a ligand containing the consensus binding sequence FPPPP (Prehoda, 1999). The WH1 domain of N-WASP has been shown to bind a proline-rich motif (DLPPPEP) of WIP similar to that recognized by the EVH1 domain of Mena (Volkman, 2002). Sequence alignment of the WH1 domain of Dictyostelium WASP with other EVH1/WH1 containing proteins revealed that the triad of residues is conserved (Fig. 1A). To investigate the role of the WH1 domain in the function of WASP, we chose to mutate the three aromatic residues that correspond to the aromatic triad: tyrosine 33, tryptophan 39, and phenylalanine 91 (Fig. 1A) to alanine. A fourth residue, phenylalanine 90, was mutated due to its proximity to F91. A construct for full length WASP carrying all four mutations (‘WASPYWFF’) was made. This construct and wild type WASP were expressed in WASP TK cells. This strain has been shown to express very low levels of WASP and exhibits strong chemotaxis defects (Myers, 2005), although random movement appears not to be significantly affected (Table I). Western analysis revealed equivalent expression of each protein, and a lack of degradation indicated that the aromatic residue mutations do not affect protein stability (data not shown). Expression of either wild type or mutant WASP rescued the ability of cells to polarize in response to cAMP pulsing; however, the WASP mutant exhibited a reduced ability to polymerize F-actin at the leading edge (Fig. 1B). Figure 1D shows that the leading edge F-actin content of WASP TK cells expressing wild type WASP (WTWASP) is three fold greater than wild type cells alone, presumably due to ectopic overexpression of WASP. However, YWFFWASP mutant exhibit less than a two-fold increase in F-actin accumulation over wild type. Consistent with this data, YWFFWASP expressing WASP TK cells exhibited a reduced level of F-actin polymerization in response to global cAMP stimulation, indicating that the aromatic triad residues are required for
full WASP function (Fig. 1C). Chemotactic parameters such as mean velocity, angular
deviation, and chemotactic index were measured. In each measurement, WASPTK cells
expressing YWFFWASP exhibited an intermediate level of stimulation between that of
WTWASP expressing cells and WASPTK cells alone, indicating WH1 domain is required
for the full function of WASP (Table I).

**WIPa, a *Dictyostelium* homolog of mammalian WIP, binds to the WHI domain of
WASP**

An attempt to identify homologues of WIP was made using the *Dictyostelium*
genome database (Fey). A gene encoding a 197 amino acid protein exhibited the highest
sequence homology to mammalian WIP and was designated WIPa (accession #
DDB0191055). A comparison of the derived amino acid sequence of *Dictyostelium*
WIPa with WIP family members from various metazoans shows a moderately conserved
primary sequence (32.2% identity with human WIP, 31.1% to human WICH, 29.9% to
rat CR16) and domain structure. WIPa is enriched in proline residues and has a
conserved WASP Homology 2 domain (WH2) at its N-terminus (Fig. 2A). This domain
has previously been reported to bind G-actin and F-actin (Ho, 2001; Martinez-Quiles,
2001; Ramesh, 1997; Vaduva, 1997).

To determine whether WIPa interacts with the WH1 domain of WASP, we
performed pull-down experiments with GST-WH1-bound glutathione beads incubated
with the lysate of cells expressing YFP-WIPa. Figure 2B shows that WTWH1, but not
YWFFWH1, pulls down WIPa from cell lysate. Coomassie staining of the membrane
revealed equal amounts of the GST fusion proteins present. To confirm that this
interaction was direct, pull down experiments were performed using purified His-tagged
WIPa. GST-\(^{WT}\)WH1- and GST-\(^{WT}\)WASP beads pulled down significantly more His-
WIPa than GST-\(^{YWFF}\)WH1 beads (Fig 2C).

We next attempted to identify the region of WIPa that interacts with the WH1
domain. Unlike human WIP, WIPa does not have a prominent WH1 binding domain
(Zetti, 2002; Volkman, 2002) at its C-terminus. One potential binding region lies in the
middle of the protein. This region has preserved the ‘LPPPP’ binding motif, as well as
conserved residues that have been suggested, using modeling data, to form electrostatic
interactions with conserved charged residues within the WH1 domain (Volkman, 2002). We mutated two of these conserved residues (K93 and S95) to alanine and performed GST pull down experiments. Figure 2D shows that the mutant WIPa (KSWIPa) exhibits a significant reduction in affinity to the WTWH1 domain. This reduction in binding averaged to 41.7\(\pm\) 8.9 % of wild type over three independent experiments.

**WIPa is enriched at the leading edge and membrane protrusions**

We previously showed localization of WASP at the leading edge of chemotaxing cells (Myers, 2005). YFP-WIPa expressed *in vivo* also localized to the leading edge of polarized, chemotaxing cells (Fig. 3A). YFP-WIPa co-localizes with CFP-WASP at the leading edge (Fig. 3C), although they do not colocalize within intracellular compartments. WIPa is transiently enriched at membrane protrusions, which appears to be an early stage of pseudopod formation (Fig. 3B). WIPa enrichment, however, seems to be lost during retraction of these protrusions, indicating that WIPa might be required for the onset of extension or maintenance of protrusion (Fig. 3A and B).

**Reduced WIPa Expression Lowers Leading Edge F-actin and Microspike Formation**

Alexa\(^{594}\)-phalloidin was used to visualize the F-actin distribution in polarized cells expressing WIPa. Both the average steady-state number of microspikes per cell, as well as the accumulation of F-actin staining at the leading edge, was significantly increased in cells overexpressing wild type WIPa (Fig. 4B, C, and D). WIPa expression in WASP\(^{TK}\) cells did not stimulate microspike formation, indicating that WIPa binding to the WH1 domain is required to stimulate microspike formation (Fig. 4B and D). To investigate the role of the WASP Homology 2 (WH2) domain of WIPa in its activity, the lysine residue 36 was mutated to alanine. This residue is conserved among WH2 domain-containing proteins (Fig. 2A) and has been shown to be critical for binding to G- or F-actin (Paunola, 2002). The K\(^{36A}\)WIPa mutant lost its ability to localize to the leading edge of chemotaxing cells. Overexpression of K\(^{36A}\)WIPa did not exhibit a significant increase in either microspike formation or F-actin accumulation(Fig. 4B, C, D), suggesting that actin binding is also required for WIPa activity.
We used RNAi technology to reduce WIPa expression [Martens, 2002]. A construct designed to express hairpin WIPa (‘hp WIPa’) RNA was electroporated into AX3 cells to reduce endogenous WIPa expression. RT-PCR analysis revealed significant reduction in the expression of WIPa mRNA, but not the messages of unrelated genes involved in F-actin regulation such as WASP, RacC and VASP (Fig. 4A). Northern analysis showed similar results (not shown). Phalloidin staining of hp WIPa cells demonstrated moderately reduced F-actin accumulation at the leading edge; however, these cells are more polarized and elongated (average length $18 \pm 0.49 \mu m$) compared to wild type (14.83 $\pm$ 0.68 $\mu m$) (Fig. 4B). These cells exhibit a lower steady-state number of microspikes along the cell body (Fig. 4D), indicating that these cells may be defective in their ability to extend lateral membrane protrusions.

**WIPa facilitates F-actin polymerization via promoting elongation of actin filaments**

Increased level of F-actin and number of microspikes in cells overexpressing WIPa suggest that WIPa stimulates F-actin polymerization. To address more specifically the role of WIPa in actin regulation during membrane extension, the barbed ends of newly formed F-actin were labeled with TRITC-G actin in cells expressing WIPa. Figure 5A shows that wild type WIPa, but not K36A-WIPa, induces an almost two-fold increase in leading edge barbed-end staining, suggesting that WIPa stimulates new actin filament formation. To support this data, *in vitro* F-actin polymerization assays were performed using high speed supernatants (HSS) prepared from cAMP-pulsed *Dictyostelium* cells as described previously (Zigmond, 1997). GTP-bound RacC binds the GTPase Binding Domain (GBD) domain of WASP and relieves its autoinhibitory interaction with the WASP acidic (A) domain (Han, 2006), presumably allowing WASP to activate the Arp2/3 complex. GTP-bound RacC initiated a roughly three-fold stimulation of actin polymerization in wild type supernatants (AX3), but not in supernatants prepared from WASP$^{TK}$ cells (Fig. 5B). Consistent with Zigmond *et al.* (1997), this small G-protein activation of actin polymerization depended upon its binding to GTP, as the inactive GDP-bound conformation of RacC failed to stimulate polymerization (AX3+GDP$^{\beta}$S RacC). Addition of 100 nM recombinant WIPa increased F-actin levels in wild type supernatant (AX3+WIPa), but not in supernatants of WASP$^{TK}$ cells (WASP$^{TK}$+WIPa).
WIPa knockdown cell supernatants (hp WIPa) exhibited a decrease and delay in the polymerization of F-actin relative to wild type supernatant. Addition of 100 nM recombinant WIPa to hp WIPa supernatants (hp WIPa+WIPa) rescued this defect (Fig. 5B). To visualize assembled F-actin, these experiments were repeated on glass cover slips and viewed with fluorescent microscopy. RacC appears to increase the number of F-actin nucleation sites, presumably via activation of WASP and the Arp2/3 complex (Figure 5C). Addition of recombinant WIPa increases the length of these actin filaments (HSS+RacC+100 nM WIPa), suggesting that WIPa promotes the elongation of actin filaments. We considered the possibility that WIPa stabilizes F-actin by preventing depolymerization of F-actin. If this were true, addition of WIPa in the presence of phalloidin stabilizing F-actin would be unlikely to lead to a further increase in the length of actin filaments. These reactions were repeated in the presence of 0.4 μM unlabeled phalloidin. The same WIPa-induced increase in the length of actin filaments was observed (data not shown), suggesting that WIPa’s activity does not depend solely upon prevention of F-actin depolymerization. WIPa addition to the supernatant in the absence of GTPγS-charged RacC does not stimulate filament formation, indicating WIPa alone does not stimulate nucleation of F-actin (data not shown).

**WIPa translocates to the cell cortex upon cAMP stimulation**

Because F-actin polymerization and subsequent membrane protrusion and chemotaxis are processes downstream of cAMP receptor activation in *Dictyostelium*, WIPa localization was monitored before and after uniform stimulation of cells with cAMP (Figure 6A). A biphasic, cortical translocation of the F-actin marker coronin and the PH<sub>CRAC</sub> domain, a marker for PI(3,4,5)P<sub>3</sub>/PI(3,4)P<sub>2</sub>, has been observed in response to uniform cAMP stimulation previously (Chen, 2003). Similarly, two phases of WIPa translocation to the cell cortex after cAMP stimulation were observed. The first phase of translocation appeared to be more uniform along the cortex and peaked after 6 seconds (Figure 6A). In contrast, the smaller second peak of WIPa translocation is less uniform and more localized to sites of membrane protrusions as PH<sub>CRAC</sub> domain does. WIPa translocation appears to require its WH2 domain, as K<sup>36A</sup>WIPa showed a significantly lower level of translocation (Figure 6A). This is consistent with the inability of this
mutant to localize to the F-actin enriched leading edge of chemotaxing cells (Fig. 3A). Similarly, WIPa expressed in WASP\textsuperscript{TK} cells exhibited a reduced ability to translocate to the cortex. These data suggest that new F-actin polymerization at the cell cortex drives WIPa translocation. To confirm this, we determined the amount of WIPa that co-sedimented with the detergent-insoluble F-actin pellet after centrifugation of lysate from cells stimulated with cAMP. Consistently, two peaks of WIPa translocation to F-actin pellet were observed. However, either mutation of the WIPa WH2 domain or WASP deletion significantly reduced the levels of WIPa in the F-actin pellet (Figure 6B).

We then measured the effects of WIPa overexpression upon the polymerization of F-actin in response to uniform stimulation of cAMP. Interestingly, WIPa leads to a significant increase in F-actin polymerization 30 seconds after cAMP stimulation, presumably at sites of membrane protrusions, while it does not significantly increase the first peak of F-actin polymerization at 5-10 sec. This WIPa-induced increase in actin polymerization is dependent upon endogenous WASP expression (Fig. 6C). PI3 kinase activity also appears to be necessary for WIPa cortical translocation, as LY294002, a PI3 kinase inhibitor, but not vehicle alone (data not shown) eliminated translocation (Fig. 6A and B). This appears to occur via a mechanism independent of actin polymerization, as LY294002 did not significantly hamper the peak increase in actin polymerization (Fig. 6C).

**WIPa is important for the ability of cells to promptly respond to a new gradient**

To determine the role of WIPa in controlling cell motility, we performed micropipette chemotaxis assays (Chung and Firtel, 1999). Polarized cells were plated on a glass slide and allowed to chemotax toward a cAMP-releasing micropipette for at least 5 minutes prior to taking images. Table 1 shows that WIPa overexpression reduces the mean velocity and chemotactic index of cells migrating towards the micropipette, while angular deviation is significantly increased, indicating these cells make more directional changes presumably due to the increase of microspike/pseudopod protrusion. These chemotactic defects are not seen in cells overexpressing \textsuperscript{K36A}WIPa (Table 1). Reduction of endogenous WIPa message, however, appears to have little effect on chemotaxis efficiency. Cells expressing the hp WIPa construct are well polarized and exhibit little
change in mean velocity and chemotactic index when migrating in a single direction towards a stably-localized micropipette (Table 1), suggesting that WIPa may not be essential for the stabilization or maintenance of a mature leading edge.

As WIPa overexpression caused frequent directional changes, we examined whether WIPa might be responsible for the ability of a cell to sense and respond to changes in the chemoattractant gradient, the micropipette was moved to a new location during chemotaxis. The instantaneous velocity of cells was measured immediately after the pipette was shifted to a location approximately perpendicular to the cell. Figure 7A shows that wild type cells redirect themselves toward the new chemoattractant source quickly. However, hp WIPa cells exhibit a delayed response to the new direction. While wild type cells resume normal velocity in less than 60 sec, hp WIPa cells took more than 300 sec to resume normal speed (Fig. 7B). We speculated that the delayed response might be due to a delay in making a new pseudopod toward the new directional cue. To test this, we measured the time required for each cell type to initiate pseudopod protrusion, in clear response to the new chemoattractant source, which eventually developed into the new leading edge (Fig. 7C). The average time required for new pseudopod formation toward the new cAMP source was 48 sec for wild type cells, while hp WIPa cells took 145 sec (Fig. 7D). These results suggest that WIPa stimulates lateral membrane protrusion and prompt formation of a new pseudopod when cells respond to a change in the direction of a chemoattractant source.

**VASP is important for WIPa-induced microspike formation**

Vasodilator-stimulated phosphoprotein (VASP) is a member of the Ena/VASP family, and targeted knockout of this gene in Dictyostelium was shown to reduce filopodia/microspike formation (Han, 2002; Schirenbeck, 2006). It is of great interest to determine if both WIPa and VASP are required for microspike formation. We expressed WIPa in a vasp null cell line. F-actin was stained to determine the steady-state level of microspikes in each cell type. Consistent with a previous study (Han, 2002), vasp null cells showed significantly low number of microspikes. Overexpression of VASP without a membrane-targeting myristoylation tag does not significantly increase the number of filopodia/microspikes per cell. vasp- cells overexpressing WIPa failed to exhibit an
increase in the steady-state level of microspikes as seen in the wild type background (Fig. 8A). Co-expression of VASP and WIPa in \textit{vasp} null cells restored increased microspike formation, suggesting that VASP might be required for microspike formation by WIPa.

As VASP has been shown to translocate to the cell cortex upon global cAMP stimulation and membrane targeting appears to be required for filopodia formation (Han, 2002), we considered the possibility that VASP may be involved in the translocation of WIPa to the cortex where it could then initiate microspike formation. Figure 8B shows a reduced WIPa translocation in the \textit{vasp} null background, raising the possibility that a lack of WIPa at the plasma membrane partially explains the lack of microspikes in the \textit{vasp} null background. VASP has been shown to nucleate and bundle actin filaments \textit{in vitro} (Schirenbeck, 2006), and as we observed a dependence of WIPa translocation upon F-actin, we measured the actin polymerization response of VASP null cells to cAMP. An increase in whole cell levels of F-actin in response to cAMP was diminished in \textit{vasp} null cells (Figure 8D). Consistently, expression of the F-actin-binding protein ABP in \textit{vasp} null cells revealed a reduced level of actin accumulation at the cell cortex (Figure 8E). Significantly less WIPa was pelleted with F-actin after cAMP stimulation of \textit{vasp} null cells, presumably due to a decrease in levels of actin polymerized and/or bundled (Figure 8C). These data suggest that, like WASP, VASP plays a role in the actin polymerization response to global cAMP. Diminished cortical actin levels in these cells lead to a decrease in the translocation of WIPa to the plasma membrane, where it might normally facilitate microspike formation with VASP.
DISCUSSION

Regulation of the actin cytoskeleton is a key event for directed cell migration. Our study suggests a role for WIpa in the regulation of the actin cytoskeleton and motility during chemotaxis. WIpa plays a role in lateral membrane protrusion and pseudopod extension that is important for prompt response to a dynamic chemoattractant gradient; whereas, WASP appears to be responsible for cell polarity and the majority of actin polymerization necessary to establish the bulk protrusive force against the leading edge plasma membrane.

Our data suggests that WIpa stimulates microspike formation and lateral membrane protrusion in a polarized cell. In these processes, WIpa may facilitate elongation of actin filaments already nucleated by WASP’s activation of the Arp2/3 complex as shown in our in vitro polymerization assay. It is also possible that WIpa may provide WASP with an additional actin monomer during Arp2/3 complex activation and enhances its rate of filament assembly. The number of WH2 domains in a complex of proteins including the Arp2/3 complex may determine the degree to which the complex enhances actin polymerization. WIpa enhances F-actin polymerization of cortactin, which has no WH2 domain (Kinley, 2003). Similarly, WASP, which has one WH2 domain and is known to be a better Arp2/3 activator than cortactin, is also stimulated by WIpa in a manner that depends upon WIpa’s own WH2 domain (Ramesh, 1997). Consistently, the VCA domain of N-WASP, which possesses two WH2 domains, was found to be a better activator of the Arp2/3 complex and microspike formation than VCA domains possessing a single WH2 domain (Yamaguchi, 2000).

NMR spectroscopy and computer modeling studies have provided insight into the pathogenesis of WAS by revealing structural details of the WASP (or N-WASP) WH1 interaction with WIpa (Kim, 2004; Rong, 2000; Volkman, 2002). Consistently, we showed that Dictyostelium WIpa binds to the WASP WH1 domain and that the aromatic triad residues are essential for this interaction. We identified a potential WH1-binding region of WIpa that lies within the middle of the protein. This region has preserved a polyproline binding motif, as well as conserved residues that have been suggested to form electrostatic interactions with conserved aspartic acid residues within the WH1 domain.
Mutation of two of these residues reduced the affinity of WIPα for the WH1 domain. This region, however, is oriented in a C- to N-terminal fashion. As proline-rich binding modules like the WH1 domain are known to isoenergetically dock with PPII ligands in two possible orientations due to the 2-fold rotational pseudosymmetry of the ligand, it is plausible that this region of WIPα interacts with the WH1 domain of WASP.

When cAMP-pulsed Dictyostelium cells are globally stimulated with chemoattractant, two distinct phases of actin polymerization are triggered. Both phases have been shown to correspond to a translocation of an F-actin marker and a PI(3,4,5)P₃ marker to the cell cortex (Chen, 2003). Cytosolic WIPα also appears to translocate to the cortical membrane in two distinct phases that parallel this actin polymerization. The reduction in translocation caused by the K36A mutation would imply that WIPα translocation is partially mediated by a WH2-F-actin interaction. Interestingly, the second phase of translocation of WIPα is localized to sites of pseudopod/microspike formation. Overexpression of WIPα would result in increased WIPα translocation to the cortex leading to more F-actin assembly (Fig. 6C) and more lateral membrane protrusion and thus lower chemotactic efficiency (Table I). WIPα translocation to the membrane also appears to require PI3 kinase activity, suggesting that PI(3,4,5)P₃ might trigger WIPα translocation. We don’t fully understand how this translocation is dependent upon PI3 kinase yet. In our previous study, WASP localization was significantly changed upon the inhibition of PI3 kinase by LY294002 (Myers et al., 2005). Sequestration of WIPα by WASP in LY294002-treated cells might result in defective translocation of WIPα to cortical membrane. We suggest that upon cAMP stimulation, membrane-bound WASP stimulates actin polymerization at the cell cortex presumably via activation by PI(3,4,5)P₃ and RacC. The polymerized actin recruits cytosolic WIPα to the cortex, where it facilitates elongation/polymerization of F-actin with WASP, resulting in membrane extension and protrusion. Cell cortical actin accumulation in response to cAMP also appears to require VASP, perhaps via its nucleation or bundling activity. A loss of this actin accumulation in VASP null cells appears to lead to a reduction in WIPα translocation similar to that seen in WASPTK cells. The reduction in cortical WIPα may be responsible for the reduced ability of WIPα overexpression to increase the steady-state level of microspikes in the absence of VASP.
We have provided evidence that WIPa regulates chemotaxis. While WIPa overexpression increases microspike formation, decreases speed, and increases angular deviation of a cell toward a chemoattractant source, reduction of WIPa expression decreases microspike formation, slightly improves the chemotactic index, but decreases the ability of cells to reorient toward a new chemoattractant source. The effects of the *Dictyostelium* formin dDia2 on cell migration are similar (Schirenbeck, 2005). Overexpression of dDia2 increases filopodium formation and leads to a decrease in cell motility. Deletion of the dDia2 gene resulted in cells with less filopodia but higher cell velocity. Because WIPa stimulates onset of early pseudopod at the plasma membrane, WIPa may allow cells to respond to new gradient promptly by initiating localized bursts of actin polymerization and/or elongation, while WASP may provide the main driving force of lamellipodia protrusion by stimulation of F-actin polymerization at the leading edge. The severe chemotactic defects exhibited by the WH1 domain WASP mutant, and the lack of significant defect of WIPa knockdown cells during chemotaxis toward a single direction, would imply that another polyproline-containing protein might participate in the stabilization or maintenance of WASP-generated actin assembly during lamellipodia protrusion at the leading edge. We have shown *in vitro* that WIPa increases the length of RacC-induced, WASP-dependent actin filaments and, *in vivo*, WIPa localizes to sites of newly forming pseudopodia on the cortical membrane. When a chemotaxing cell senses a change in direction in cAMP input, a localized increase in WIPa activity might provide the cytoskeletal framework for the formation of a new pseudopod and, eventually, a new leading edge by facilitating actin polymerization. A decrease in WIPa expression, then, would result in cells with a reduced ability to change direction promptly upon change of chemoattractant gradient due to delayed formation of pseudopodia/microspikes. It is likely that chemotaxis of these cells already committed towards a single chemoattractant source would not be altered or even faster due to less angular deviation. An increase in WIPa expression would produce more lateral pseudopodia resulting in a corresponding increase in the number of turns, and thus a greater angular deviation. A role for both WIP and WASP in *Dictyostelium* chemotaxis is consistent with the severe impairment of T cell homing to spleen and lymph nodes from WASP<sup>−/−</sup>WIP<sup>+/+</sup> double knockout mice (Gallego, 2005).
A species-specific difference, however, between the importance of the WASP family of proteins may exist. While lamellipodia and filopodia formation appear to be more dependent upon SCAR in Drosophila BG2 cells (Biyasheva et al., 2004) and WAVE2 in mammalian cells (Yan et al., 2003), WASP deficiency appears to have a greater effect on membrane protrusion and actin assembly than SCAR deficiency in Dictyostelium. SCAR deficiency does lead to decreased actin assembly at the leading edge (Bear et al., 1998), but these cells polarize well and form visible streams, unlike WASP<sup>TK</sup> cells, suggesting the possibility of rather normal chemotactic movement despite abnormal F-actin organization. It has been suggested that SCAR is not required for the generation of actin protrusions, but may be more involved in extending and reshaping them (Blagg, 2004). This would imply the evolutionary acquisition of WASP-dependent roles of actin assembly during chemotaxis by WAVE/SCAR family members sometime after the divergence of the protozoans. Alternatively, it is conceivable that WASP function is more essential in highly motile cells such as Dictyostelium, neutrophils, and macrophage to provide highly dynamic regulation of the actin cytoskeleton than in slower moving cells like fibroblasts. It has been demonstrated that the VCA domain of SCAR shows different potency in stimulating Arp2/3 than that of WASP or N-WASP, at least in <em>in vitro</em> systems. The VCA domain of N-WASP and WASP induce rapid actin polymerization, while SCAR1 induces the slowest rate of nucleation (Zalevsky <em>et al.</em>, 2001). It is conceivable to expect WASP or N-WASP to be the primary regulators of dynamic F-actin assembly. Thus, lack of WASP function might cause more severe phenotypes in highly motile cells.

In conclusion, WIPa translocates from the cytoplasm to the plasma membrane upon cAMP stimulation. The regulation of the F-actin cytoskeleton by WIPa at sites of membrane protrusion and pseudopod formation appears necessary to allow chemotaxing cells the flexibility of responding to new sources of chemoattractant and to prevent premature commitment to a single chemoattractant source.
Materials and Methods

Cell Culture and Development

*Dictyostelium* cells were cultured axenically in HL5 medium supplemented with 60 units of penicillin and 60 μg of streptomycin per ml. All cell lines shown were obtained using G418 to select stable-expressing clones. Equivalent protein expression levels were determined via quantitation of YFP signal *in vivo* using Metamorph™ software. For examining developmental phenotypes, cells were washed twice with 12 μM Na/K phosphate buffer and plated on non-nutrient agar plates.

Molecular Biology

WASP and WIP expression constructs were produced by PCR amplification of *Dictyostelium* cDNA library or genomic DNA and subcloning into the pEXP4(+) vector in frame with eYFP at the N-terminus separated by the flexible linker GSGSG. The YFP fusion proteins were expressed under control of the actin-15 promoter. Point mutants were produced by cloning genes into pBS, using the QuikChange site-directed mutagenesis kit (Stratagene), and subcloning into pEXP4(+). All mutants were confirmed by sequence analysis. Protein expression constructs were produced using pGEX6P-1 (GST-tag) or pET-32a+ (6xHis-tag). Hairpin WIPα RNA construct was made by amplifying full-length WIPα and C-terminal half of WIPα and cloning them into pEXP4(+) in a tail to tail manner.

*In vitro* Pull down Assay

Protein expression constructs produced using pGEX6P1 or pET-32a+ were transformed into BL21 cells. Cultures were grown to a cell density of OD₆⁰₀ = 0.8, and 0.1 mM IPTG was added to induce protein expression for 1 hour @ 37°C. Proteins were purified according to the manufacturers’ instructions (Sigma-GST proteins; Qiagen-His proteins). Equal quantities of recombinant protein were added to 500μl TBS-T containing 60μl of GST-tagged protein bound to glutathione-conjugated agarose beads and incubated at 4°C for 1 hour. The agarose beads were pelleted by centrifugation at 14,000 rpm for 5 min and washed 3x with 1 ml TBS-T. Protein was then stripped off the beads in 2x sample buffer by boiling for 4 min. Samples were resolved on a 12% SDS-PAGE gel, and proteins were identified via western analysis using a mouse anti-6x His antibody (Cell Signaling). *In vivo* pull down experiments were similarly performed, but agarose beads were incubated in 100μl cell lysate, and proteins were identified using a mouse anti-GFP antibody.

*In vitro* Actin Polymerization Assay

Experiments were performed as described by (Zigmond, 1997). High-speed supernatants (HSS) were prepared by pulsing cells with 30 nM cAMP at 6 min. intervals for 5 hr, shaking with 3 mM caffeine for 30 min., lysing cells by passage through a 5 μM pore filter in PM buffer (12 mM Na/K phosphate buffer, pH 6.1, 2 mM MgSO₄, plus 20 mM KCl and 1 mM EGTA), spinning for 20 min. @ 2.8e⁵ x g, and freezing in
liquid nitrogen. One hundred nM purified RacC was charged with either 100 μM GTPγS or 100 μM GDPβS (Φ) for 10 min. @ 30°C. Supernatants were then stimulated at room temperature with the nucleotide-bound RacC at time 0. 50 μl aliquots were taken at each time point and stopped by dilution into 850 μl of actin buffer containing 6% formaldehyde, 0.15% Triton X-100, 1 μM TRITC-phalloidin. Actin was stained for 1 hr. at room temperature and spun down at 14,000 rpm for 10 min. Rhodamine-phalloidin was extracted with 300 μl of 100% methanol and fluorescence was measured (540ex/575em).

For visualization of actin filaments, supernatants were allowed to polymerize in the presence or absence of 0.4 μM phalloidin on a slide cover slip for 5 min. at 37°C before stopping reactions with actin buffer containing formaldehyde and TRITC-phalloidin. Images were taken using fluorescent microscopy with a 60x objective lens and Metamorph™ software.

**F-Actin Pull down Assay**

Experiments were performed the same as *in vivo* actin polymerization assays, except 100 μl of cells were mixed at each time point in Dicty. lysis buffer (25 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, 1 mM DTT, 3 μM phalloidin). Lysates were spun down at 14,000 rpm for 10 min. Pellets were boiled in 2x loading buffer, resolved on 10% SDS-PAGE, blotted onto nitrocellulose, and YFP-WIPα was detected using an anti-GFP antibody.

**F-Actin Staining**

For phalloidin staining, cells were pulsed with 30 nM cAMP at 6 min. intervals for 5 hr. and placed on glass cover slips in 12 mM sodium phosphate buffer (pH 6.2) + 200 μM each of MgCl₂ and CaCl₂ for at least 30 min. Cells were fixed with 3.7% formaldehyde for 10 min. Cells were permeabilized with 0.5% Triton X-100, washed, and incubated with TRITC or Alexa™-phalloidin (Sigma) in PBS containing 0.5% BSA and 0.05% Tween-20 for 30 min. Cells were washed in PBS containing 0.5% Tween-20. Images were captured with a Roper Coolsnap camera and Metamorph™ software.

For labeling barbed-ends, cells were permeabilized with 100 mM PIPES, pH 6.9, 1% Triton X-100, 4% PEG, 1 mM EGTA, 1 mM MgCl₂, 3 μM phalloidin for 3 min. and 0.4 μM Rhodamine-labeled G-actin in 1 μM ATP solution was added. After 5 min. staining, cells were washed 3x with PIPES buffer and fixed with 3.7% paraformaldehyde. For quantitation of leading edge F-actin content, the amount of F-actin at the leading edge of these cells was quantitated by measuring the integrated phalloidin signal (area x average intensity) at the leading edge divided by the average intensity of phalloidin staining throughout the cell body.

**In vitro Actin Polymerization Assay**

Experiments were performed as described by (Zigmond, 1997). High-speed supernatants (HSS) were prepared by pulsing cells with 30 nM cAMP at 6 min. intervals for 5 hr, shaking with 3 mM caffeine for 30 min., lysing cells by passage through a 5 μM pore filter in PM buffer (12 mM Na/K phosphate buffer, pH
6.1, 2 mM MgSO₄, plus 20 mM KCl and 1 mM EGTA), spinning for 20 min. @ 2.8e⁵ x g, and freezing in liquid nitrogen. One hundred nM purified RacC was charged with either 100 μM GTPγS or 100 μM GDPβS for 10 min. @ 30°C. Supernatants were then stimulated at room temperature with the charged RacC at time 0. 50 μl aliquots were taken at each time point and stopped by dilution into 850 μl of actin buffer containing 6% formaldehyde, 0.15% Triton X-100, 1 μM TRITC-phalloidin. Actin was stained for 1 hr. at room temperature and spun down at 14,000 rpm for 10 min. Rhodamine-phalloidin was extracted with 300 μl of 100% methanol and fluorescence was measured (540ex/575em).

For visualization of actin filaments, supernatants were allowed to polymerize in the presence or absence of 0.4 μM phalloidin on a slide cover slip for 5 min. at 37°C before stopping reactions with actin buffer containing formaldehyde and TRITC-phalloidin. Images were taken using fluorescent microscopy with a 60x objective lens and Metamorph™ software.

**Chemotaxis Assay**

Cells were pulsed with 30 nM cAMP at 6 min. intervals for 5 hr. These conditions maximally induce expression of aggregation-stage genes, including the cAMP receptor cAR1 and the coupled G protein α subunit Ga2. The chemotaxis assays were performed as previously described (Chung, 1999), (Meili, 1999). After pulsing, cells were plated on glass-bottomed microwell dishes (MarTek Inc., Ashland, MA). A micropipette filled with 100 μM cAMP was positioned to stimulate cells by using a micromanipulator (Eppendorf, Germany) and the response and movement of cells were recorded by using Metamorph™ software (1 image per every 5 sec). Cell movement was examined by tracing the movement of a single cell in a stack of images.

**Subcellular Localization and Fluorescent Microscopy**

Cells were pulsed as normal and plated onto glass-bottomed microwell dishes. Cells were stimulated with 100 mM cAMP either using a micropipette or globally with an Eppendorf pipetman. YFP localization and translocation were recorded by using Metamorph™ software (1 image per every 3 sec) and a YFP emission filter. Quantitation of YFP signal was performed using line scan of lines transecting the cell body for each time point before and after stimulation.
Fig. 1. **Conserved WH1 domain aromatic residues are important for WASP function.** (A) Comparison of the amino acid sequence of DdWASP WH1 domain with other species [(Hs) *Homo sapiens*; (Mm) *Mus musculus*; (Ce) *Caenorhabditis elegans*; (Dm) *Drosophila melanogaster*; (Dd) *Dictyostelium discoideum*]. Asterisks indicate residues with naturally occurring mutations in WASP patients. Arrows indicate residues mutated to alanine. Predicted secondary structure is indicated below sequence. (B) *In vivo* F-actin organization as determined by Alexa^594^-phalloidin staining. WH1 domain mutations (YWFF) reduce F-actin levels at leading edge of WASP^TK^ cells rescued via ectopic WASP expression. Equivalent expression levels of WASP were determined via quantitation of YFP fluorescence and western analysis. Bar = 5µm. (C) *In vivo* actin polymerization assay. Full rescue of WASP^TK^ phenotype by WASP overexpression requires WH1 aromatic residues. (D) Quantitation of F-actin at the leading edge. F-actin levels were determined by measuring the integrated Alexa^594^-phalloidin signal intensity (area x average intensity) at the leading edge divided by the average intensity of phalloidin staining throughout the cell body. A minimum of 100 cells of each type was taken from at least three independent experiments.

Fig. 2. **DdWIPa is structurally and functionally similar to mammalian Wasp Interacting Protein (WIP).** (A) Schematic representations of mammalian WIP and *Dictyostelium* WIPa. Sequence alignment of Wasp Homology 2 (WH2) domain shown with conserved residues in black. Residues essential for binding to G-actin are indicated with asterisks. Lysine 36 is indicated with an arrow. (B) YFP-WIPa was precipitated from AX3 cell lysates with either GST-fusion wild type or mutant WH1 bound to glutathione-beads. Representative of three experiments is shown. IB: immunoblot; C: coomassie staining. (C) Purified His-tagged WIPa was pulled down from solution using GST-WH1 or -WASP bound to beads. Representative of three experiments is shown. Each pulldown was quantitated and normalized to the quantity of GST-tagged protein used in the experiment. (D) Interaction between WH1 domain and WIPa depends partially upon conserved residues K93 and S95 of WIPa. Purified His-tagged WT or mutant ‘KS’ WIPa was pulled down from solution using GST-WH1. Representative of three experiments is shown.

Fig. 3. **WIPa localizes to leading edge, sites of membrane protrusion, and colocalizes with WASP.** (A) WIPa localization in chemotaxing cells. YFP-tagged WIPa, but not actin-binding domain mutant K36A WIPa, is enriched at the leading edge of polarized cells. (B) Transient WIPa localization at sites of membrane protrusion. Images were taken at 6 sec intervals. (C) YFP-tagged WIPa colocalizes with CFP-tagged WASP at the leading edge of chemotaxing cells. Asterick indicates location of micropipette tip releasing 100 µM cAMP.

Fig. 4. **F-actin accumulation and filopodia formation are induced by WIPa.** (A) Expression of hairpin WIPa ‘hp WIPa’ construct reduces WIPa mRNA level. RT-PCR reactions were performed using mRNA purified from 5 hour pulsed *Dictyostelium* cells. Expression of other genes essential for F-actin regulation was unaffected. (B) F-actin organization in cells expressing hpWIPa or WIPa mutants. At least 150 cells taken from three independent experiments were used for quantitation of (C) leading edge F-actin.
content and (D) filopodia number. Quantitation of in vivo YFP signal revealed equivalent levels of WIPa expression in each cell type. ‘WIPa-WASP<sup>TK</sup>’ refers to the overexpression of wild type WIPa in the WASP<sup>TK</sup> background.

**Fig. 5.** WIPa stimulates de novo F-actin polymerization. (A) Barbed-end staining of cells expressing wild type WIPa or mutant (K36A WIPa). At least 60 cells taken from three independent experiments were used for quantitation. (B) In vitro F-actin polymerization assay. Supernatants of either wild type AX3, WASP<sup>TK</sup>, or hp WIPa cells were stimulated with GTPγS-bound RacC at time 0 (or with GDPβS-bound RacC as indicated ◊). 100 nM WIPa was added 15-30 sec after RacC addition where indicated. Reactions were stopped at the indicated time points with actin buffer containing TRITC-phallloidin, and F-actin was pelleted by centrifugation. The amount of F-actin at time 0 was standardized as 100%. (C) Visualization of polymerized F-actin. Supernatants were incubated with or without 100 nM GTPγS-charged RacC or WIPa for 5 minutes on a poly-L-lysine treated glass cover slip and then fixed.

**Fig. 6.** WIPa translocates to the cell cortex upon global cAMP stimulation. (A) Images of cells expressing YFP-WT WIPa or K36A mutant globally stimulated with 100 μM cAMP shown. Data points were taken at 3 sec intervals. Two linescan measurements from at least 24 cells of each cell type were used for quantitation of YFP intensity at the membrane. (B) F-actin pull-down of YFP-WIPa. WIPa interaction with F-actin depends upon WH2 domain and PI3 kinase activity. (C) In vivo F-actin polymerization assay. WT WIPa overexpression leads to an increase in actin polymerization 30 seconds after global cAMP stimulation.

**Fig. 7.** WIPa is necessary for prompt response to a changing chemoattractant gradient. (A) Pulsed cells were allowed to chemotax toward a cAMP-releasing micropipette (*) for about 5 minutes. Time 0 indicates the first frame after which the micropipette was moved to its new position. Time-lapsed images were taken at 5 sec intervals for a total of about 90 frames. Chemotactic parameters of each cell type were averaged from 3 independent experiments (AX3 = 35 cells total; hp WIPa = 38 cells total). (B) Instantaneous velocity of each cell type after micropipette shift as tracked by Metamorph<sup>TM</sup>. Averages of 10 cells of each type from three independent experiments are shown. (C) hp WIPa cells require more time to initiate membrane protrusion, and subsequently pseudopodia formation, toward the direction of the new gradient. (D) The average time required for forming new pseudopodia toward the new cAMP source.

**Fig. 8.** VASP is required for WIPa-induced microspike formation and membrane translocation. (A) Overexpression of WIPa fails to significantly increase the steady-state level of microspikes in a vasp null background. The number of microspikes from at least 50 cells was counted in each of three independent experiments. (B) YFP-WIPa translocation. Significantly less WIPa translocates to the cell cortex upon cAMP stimulation in the absence of VASP. (C) F-actin pulldown of YFP-WIPa is decreased in VASP null cells (D) In vivo F-actin polymerization in response to cAMP is defective in VASP-null cells. (E) Cortical translocation of the F-actin marker GFP-ABP in response to global cAMP stimulation is defective in VASP null cells.
REFERENCES


**TABLE I**
Chemotactic parameters

<table>
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<tr>
<th>Strain</th>
<th>MeanVelocity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Angular Deviation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Chemotactic Index&lt;sup&gt;c&lt;/sup&gt;</th>
<th>cell number</th>
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<tr>
<td>AX3 wildtype</td>
<td>8.40 ± 2.10</td>
<td>42.91 ± 7.14</td>
<td>0.58 ± 0.19</td>
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<td>WT WIPα / AX3</td>
<td>6.21 ± 1.75*</td>
<td>55.57 ± 8.82*</td>
<td>0.35 ± 0.38*</td>
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<td>K36A WIPα / AX3</td>
<td>8.58 ± 3.20</td>
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<td>WT WIPα / WASP&lt;sup&gt;TK&lt;/sup&gt;</td>
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<td>67.12 ± 7.38*</td>
<td>0.03 ± 0.02*</td>
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<tr>
<td>bp WIPα</td>
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<td>40.28 ± 7.21*</td>
<td>0.66 ± 0.13*</td>
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<tr>
<td>WT WASP / WASP&lt;sup&gt;TK&lt;/sup&gt;</td>
<td>6.94 ± 2.92</td>
<td>54.92 ± 11.77</td>
<td>0.44 ± 0.21</td>
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<td>YWFF WASP / WASP&lt;sup&gt;TK&lt;/sup&gt;</td>
<td>4.51 ± 2.64*</td>
<td>62.27 ± 11.15*</td>
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<td>-0.02 ± 0.08*</td>
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<td>Random Movement</td>
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<td>AX3</td>
<td>3.27 ± 0.71</td>
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<td>4.13 ± 0.55</td>
<td>66.6 ± 3.11</td>
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</table>

<sup>a</sup> Mean velocity indicates speed of cell’s centroid movement along the total path.

<sup>b</sup> Angular Deviation indicates an average of the deviations of the angle of the path taken by cells from one frame to the next.

<sup>c</sup> Chemotaxis index is calculated as described in Futrelle et al. (1982). If the cell moves directly toward the gradient source it is 1, if directly away it is -1. If movements are indifferent to gradient (random movement), it is 0.

nd = not determined.

* p values were computed by Student’s t test, and values below 0.05 were considered significant.