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A Complex of Wiskott-Aldrich Syndrome Protein with Mammalian Verprolins Plays an Important Role in Monocyte Chemotaxis[1]

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The Wiskott-Aldrich syndrome protein (WASP) is a product of the gene defective in an Xid disorder, Wiskott-Aldrich syndrome. WASP expression is limited to hemopoietic cells, and WASP regulates the actin cytoskeleton. It has been reported that monocytes/macrophages from WASP-deficient Wiskott-Aldrich syndrome patients are severely defective in chemotaxis, resulting in recurrent infection. However, the molecular basis of such chemotactic defects is not understood. Recently, the WASP N-terminal region was found to bind to the three mammalian verprolin homologs: WASP interacting protein (WIP); WIP and CR16 homologous protein (WICH)/WIP-related protein (WIRE); and CR16. Verprolin was originally found to play an important role in the regulation of actin cytoskeleton in yeast. We have shown that WASP, WIP, and WICH/WIRE are expressed predominantly in the human monocyte cell line THP-1 and that WIP and WICH/WIRE are involved in monocyte chemotaxis. When WASP binding to verprolins was blocked, chemotactic migration of monocytes was impaired in both THP-1 cells and primary human monocytes. Increased expression of WASP and WIP enhanced monocyte chemotaxis. Blocking WASP binding to verprolins impaired cell polarization but not actin polymerization. These results indicate that a complex of WASP with mammalian verprolins plays an important role in chemotaxis of monocytes. Our results suggest that WASP and mammalian verprolins function as a unit in monocyte chemotaxis and that the activity of this unit is critical to establish cell polarization. In addition, our results also indicate that the WASP-verprolin complex is involved in other functions such as podosome formation and phagocytosis. ***The Journal of Immunology,*** **2006, 176: 6576–6585.**

The Wiskott-Aldrich syndrome (WAS)[3] is an X-linked recessive condition described as a clinical triad of immunodeficiency, thrombocytopenia, and eczema. Without aggressive treatment such as bone marrow transplantation, most patients die by age 10 due to recurrent infections, hemorrhage, or autoimmune diseases (1–4). The causative gene underlying WAS encodes the Wiskott-Aldrich syndrome protein (WASP) (5), which plays an important role in hemopoietic cells in the assembly of actin cytoskeleton (6, 7), in signal transduction (8, 9), and in apoptosis (10, 11).

WASP contains several domains that interact with diverse cellular molecules (4) (see Fig. 1*A*). Binding of phosphatidylinositol 4,5-bisphosphate and Cdc42 to the basic region and the GTPase binding domain disrupts the autoinhibited WASP conformation, resulting in activation of the WASP C-terminal region (verprolin/cofilin/acidic (VCA) domain) (see Fig. 1*A*). The VCA domain of WASP stimulates actin polymerization by interacting with an actin-related protein complex, the Arp2/3 complex (12, 13). Other molecules that bind to WASP to activate the VCA domain such as vasodilator stimulated phosphoprotein and Nck have also been reported (14–19). Recently, the Toca-1 protein (transducer of Cdc42-dependent actin assembly) was identified as a crucial intermediate required for Cdc42-neural WASP (N-WASP)-Arp2/3 complex-induced actin polymerization in cell extracts (20).

A homologous protein, N-WASP, has the same domain organization as WASP (see Fig. 1*A*). WASP is expressed predominantly in hemopoietic cells, whereas N-WASP is ubiquitously expressed, but rich in neural cells (21).

In contrast to the well-characterized WASP C-terminal function, little is known about the WASP N-terminal region. Verprolin was originally identified as a yeast protein that is implicated in cell growth, cytoskeletal organization, and endocytosis (22). Three mammalian verprolins have been identified as proteins binding to the WASP N-terminal region (residues 1–170), termed WASP interacting protein (WIP) (23); WIP and CR16 homologous protein (WICH) (24) or WIP-related protein (WIRE) (25); and CR16 (26) (see Fig. 1*B*). WICH/WIRE was identified simultaneously by two independent groups. Mammalian verprolins, WIP, WICH/WIRE, and CR16 have the same domain organization consisting of the verprolin homologous (V_H) domain, a proline-rich region (Pro-rich), and a WASP binding domain (WB) (see Fig. 1*B*). WIP can suppress the defects of verprolin-deficient yeast (27), suggesting that the V_H domain of WIP has the same function as verprolin in yeast.

WIP is widely expressed and crucial for localizing WASP activity both in a vaccinia-based actin motility system and to the immune synapse after TCR ligation (28, 29). In addition, WIP synergizes with N-WASP to induce filopodia when overexpressed in fibroblast. The structure of the N-WASP-WIP complex has been

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[3] Abbreviations used in this paper: WAS, Wiskott-Aldrich syndrome; WASP, Wiskott-Aldrich syndrome protein; VCA, verprolin/cofilin/acidic; N-WASP, neural WASP; WIP, WASP interacting protein; WICH, WIP and CR16 homologous protein; WIRE, WIP-related protein; WB, WASP binding domain; SDF-1α, stromal cell-derived factor-1α; siRNA, small interfering RNA; X-linked thrombocytopenia (XLT); EGFP, enhanced GFP.

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solved, and it gave us an insight into the molecular basis of WAS (30).

WICH induces actin-microspike formation through interaction with N-WASP in WICH overexpressing cells and has actin-bundling activity (24, 31). WIRE induces platelet-derived growth factor-mediated membrane protrusions by cooperating with WASP in WIRE overexpressing cells and plays a role in platelet-derived growth factor receptor endocytosis (25, 32). CR16 was identified as a glucocorticoid-enhanced gene product and is expressed predominantly in the brain. CR16 and N-WASP colocalize and function in primary hippocampal neuron (26, 33).

The most common causes of death of WAS patients are hemorrhage, recurrent infection, and autoimmune diseases, although the disease mechanisms are not well understood. Recurrent infection is suggested to be due to chemotactic defects in hemopoietic cells such as neutrophils (34–39), monocytes/macrophages (34, 35, 37, 38, 40–43), and dendritic cells (37, 40, 43–47). However, the molecular mechanisms underlying chemotactic defects are unclear. Here, we investigate the roles of the WASP-verprolin complex in chemotaxis of monocytes.

Materials and Methods

Reagents

Human MCP-1 and stromal cell-derived factor-1α (SDF-1α) were purchased from R&D Systems. Anti-WASP mAb and anti-WIP polyclonal Ab were purchased from Santa Cruz Biotechnology. PMSF, leupeptin, pepstatin A, aprotinin, and anti-mouse IgG agarose were obtained from Sigma-Aldrich. pEGFP-C2, pHcRed-C1, anti-EGFP mAb, and anti-HcRed polyclonal Ab were from BD Clontech. RPMI 1640 and other tissue culture reagents were obtained from Invitrogen. A polyclonal Ab to WICH/WIRE was prepared in this study. The synthetic peptide of WICH/WIRE, NH_2-SNKAQAYNREKPLPPTPGQ-COOH (residues 197–215) (Anaspec) was used to immunize rabbits. The Ab was purified by affinity chromatography using the synthetic peptide coupled to Sepharose beads.

Cell culture and transfection

Human monocyte cell lines, THP-1 and U-937, were obtained from American Type Culture Collection and cultured in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. All transient transfection studies were performed using the FuGene6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. The N-terminally EGFP-tagged WASP-binding domain of WIP (EGFP-WB) or EGFP only (EGFP) was expressed in THP-1 cells using the pEGFP-C2 expression vector. Two days after transfection, EGFP-positive THP-1 cells were sorted by a FACS, FACSDiva (BD Immunocytometry System), and the sorted cells were used for immunoprecipitation, a chemotaxis assay, and a polarization assay on the same day. HcRed-positive THP-1 cells were prepared in an identical manner. Transfection of small interfering RNA (siRNA) was performed using Oligofectamine (Invitrogen) in Opti-MEM medium. siRNAs were purchased from Dharmacon. The following target sequences were chosen to generate siRNA: for WASP, 5′-CGAGA ACCAGCGACTCTTT-3′ (sense), 5′-TCCAGACCTTGCAATCGGC-3′ (scrambled); for WIP, 5′- GATCCACATCTGCGAAACC-3′ (sense), 5′-AACCTCGGAGCCTCAACTA-3′ (scrambled); for WICH/WIRE, 5′-GAGAACCTAGCTGGTAAGC-3′ (sense), 5′-CACCAGCATTGGACA TGGA-3′ (scrambled). The efficiency of siRNA transfection measured using FITC-conjugated control siRNA (Santa Cruz Biotechnology) was 60–80%.

Human monocyte isolation and transfection

After informed consent was obtained, 40 ml of peripheral blood was drawn from healthy volunteers. Monocytes were isolated from blood samples using RosetteSep and FicollPaque (StemCell Technologies). Freshly isolated monocytes were transfected with EGFP or EGFP-WB constructs using Human Monocyte Nucleofector kit and amaxa Nucleofector (amaxa) according to the manufacturer's instructions.

Recombinant proteins

The WASP binding domains of WIP (residues 321–503) and WICH/WIRE (residues 320–440) were expressed in *Escherichia coli* (BL21DE) as GST fusion protein (GST-WIPWB and GST-C/RWB) and purified from bacterial lysates by batch elution from glutathione-Sepharose. WASP was expressed as N-terminally Myc-tagged proteins in human embryonic kidney (HEK) 293 cells and prepared from 293 cell transfectants using anti-Myc (9E10)-conjugated Sepharose (Santa Cruz Biotechnology).

Surface plasmon resonance

A Biacore 3000 (Biacore) surface plasmon resonance-based biosensor was used to determine affinity constants for the binding of WASP to WIP and WICH/WIRE. Purified GST-WIPWB and GST-C/RWB were immobilized onto the carboxymethylated dextran surface of a CM5 sensor chip using the amino-coupling kit (Biacore). Myc-WASP was diluted in running buffer (10 mM HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) polysorbate 20) and injected onto the sensor chip at varying concentrations. Analyte was injected at concentrations of 250, 125, 62.5, 37.5, 18.8, and 9.38 nM. Specific interaction data were first subtracted from corresponding controls, zeroed using the BIAevaluation 2.0 software (Biacore), and then globally fit to a simple bimolecular reaction model using Clamp software. The analysis was performed three times independently. The dissociation affinity constants (K_D) of WIP and WICH/WIRE are defined as $K_D = k_{off}/k_{on}$ and estimated using the BIAevaluation 2.0 software.

Immunoprecipitation

For coimmunoprecipitation of WASP with WIP or WICH/WIRE, THP-1 cells (5×10^7 cells) were lysed in buffer A (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% IGEPAL CA-630 (Sigma-Aldrich), 1 mM PMSF, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin). Lysates were centrifuged at 10,000 $\times$ *g* at 4°C for 15 min. The supernatant was incubated with 2 μg/ml mAb to WASP (Santa Cruz Biotechnology) at 4°C for 2 h. The agarose resin binding the immune complex was washed with 0.5 ml of buffer A three times, and the complex was diluted with 1× Laemmli's SDS-PAGE sample buffer. Eluted proteins were subjected to SDS-PAGE and analyzed by immunoblotting using anti-WIP polyclonal Ab (Santa Cruz Biotechnology) and anti-WICH/WIRE polyclonal Ab.

Chemotaxis assay

Migration of THP-1 and U-937 cells was determined using a 24-Transwell apparatus (6.5 mm diameter, 5 μm pore size) (Corning). Cells were resuspended in migration buffer (0.1% BSA/RPMI serum-free medium) to adjust cell density to 1×10^6 cells/ml. One hundred microliters of suspension (1×10^5 cells) was added to the top chamber of the 24-Transwell and 600 μl of migration buffer containing chemokines was added to the lower chamber, and incubated at 37°C for 2 h in an atmosphere containing 5% CO_2 (48). Cells that passed through the membrane were collected from the lower well and counted in a flow cytometer, FACSort (BD Biosciences).

RNA isolation and RT-PCR

Total RNA was isolated with PureLink 96 Total RNA Purification kit (Invitrogen) according to the manufacturer's instructions. Human adult brain total RNA was purchased from Stratagene. After reverse transcription of 2 μg of total RNA by oligo(dT) priming, the resulting single-strand cDNA was amplified using Expand High Fidelity PCR system (Roche Applied Science). PCR primers used were the following: β-actin sense (5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′), β-actin antisense (5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′), WASP sense (5′-TGGACCTAGCCCAGCTGATA-3′), WASP antisense (5′-AGGGG TCTTGTTCAGCTGA-3′), N-WASP sense (5′-GTCAACAACATCTC CCATACC-3′), N-WASP antisense (5′-TCTTGGCCATCAGCCAC AGA-3′), WIP sense (5′-TCAGTTGCCATCCAGGAGTG-3′), WIP antisense (5′-TTGGATCCACTCCGGCTTTC-3′), WICH/WIRE sense (5′-ACCTCGGCCTCCCCATCTTTA-3′), WICH/WIRE antisense (5′-GAAAGACCGGACAGTGGTGATA-3′), CR16 sense (5′-AAGTCCC CCAGCTTCCAGA-3′), and CR16 antisense (5′-GCCTTTAGAGATA ACTGAG-3′). PCR was done on 100 ng of single-stranded cDNA in the presence of 5 μM each oligonucleotide primer in an Applied Biosystems 2720 Thermal Cycler (40 cycles, denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min). Aliquots of 10 μl of the amplification products were separated by 1.0% agarose gel electrophoresis, visualized by ethidium bromide staining, and quantified by Alpha Imager analysis.

Determination of cellular F-actin and cell polarization

THP-1 cells were suspended at 2×10^6 cells/ml in RPMI 1640 medium supplemented with 2% (w/v) BSA (Sigma-Aldrich) and 10 mM HEPES (pH 7.2) and preincubated for 2 h at 37°C. After addition of MCP-1 (10 nM), cells were incubated for 15–30 min at 37°C. THP-1 cells were fixed

by adding an equal volume of 8% (w/v) paraformaldehyde (Fluka) in PBS. Wet preparations were visually scored by phase-contrast microscopy for cell polarization. Two hundred cells were examined. To determine F-actin content, fixed cells were permeabilized and stained in a single step with 0.1% (w/v) Triton X-100 and 5 μg/ml Alexa 488-phalloidin (Molecular Probes), and analyzed by FACS.

Podosome staining

THP-1 cells were stimulated with 50 ng/ml PMA for 3 days, and cells were seeded on coverslips. Cells were fixed with 4% paraformaldehyde (Fluka), permeabilized with 0.1% Triton X-100, and then stained with Alexa 568-phalloidin (Molecular Probes) to visualize actin core in podosomes. Podosome staining was examined under a fluorescence microscope (Zeiss Exoplan AR).

Phagocytosis assay

Fluorescence-labeled latex beads (0.75 μm Fluoresbrite Yellow Green Carboxylate Microspheres; Polysciences) were opsonized with 10% unheated FBS for 60 min at 37°C. PMA-stimulated THP-1 cells were seeded on coverslips and incubated with fluorescence-labeled latex beads at the final concentration of 0.0027% (v/v) in complete RPMI 1640 medium containing 10% FCS for 8 h at 37°C in a CO_2 incubator. Control plates were incubated at 4°C to estimate nonspecific binding of microspheres to the cells. Uptake of fluorescent beads was quantified by counting a fluorescence microscope. The percent phagocytosis was calculated as a percentage of the number of cells with counting at least one bead of the total number of cells counted. At least 200 cells were examined.

Statistical analysis

The significance of differences between groups was calculated by Student's *t* test. Confidence (95%) was set a priori as the desired level of statistical significance.

Results

Expression of WASP family proteins and mammalian verprolins in monocytes

There are two WASP homologous proteins, WASP and N-WASP, and three mammalian homologs of verprolin, WIP, WICH/WIRE, and CR16 (23–26). To determine which ones are expressed in human monocytes, mRNA expression of those five proteins in a human monocyte cell line, THP-1 cells, was analyzed by RT-PCR. WASP was expressed predominantly in THP-1 cells, and N-WASP was expressed predominantly in human adult brain (Fig. 1*C*). In contrast, WIP and WICH/WIRE were expressed abundantly in both THP-1 and brain as a verprolin (Fig. 1*C*). These results suggest that WASP, WIP, and WICH/WIRE physiologically function in THP-1 cells.

The expression levels of these proteins in two human monocyte cell lines, THP-1 and U-937, were examined by immunoblotting. All three proteins, WASP, WIP, and WICH/WIRE, were detected in THP-1 cell lysates, but in U-937 cell lysates, WASP was not detected and WIP expression levels were much lower than seen in THP-1 cell (Fig. 1*D*). Thus, to investigate the function of WASP family and mammalian verprolins in human monocytes, THP-1 cells expressing WASP, WIP, and WICH/WIRE were primarily used in the experiments described below.

Characterization of WASP binding to mammalian verprolins

To further characterize binding of WASP to WIP or WICH/WIRE expressed in monocytes, binding was analyzed using a surface plasmon resonance-based biosensor, Biacore 3000 (Biacore). The WASP-binding domains of WIP (residues 321–503) and WICH/WIRE (residues 320–440) were prepared as GST-fusion proteins, designated as GST-WIPWB and GST-C/RWB, respectively. A binding signal was recorded, when Myc-tagged WASP (Myc-WASP) was passed over a sensor chip with immobilized GST-WIPWB (Fig. 2*A*, *top*). A binding signal was also recorded, when Myc-WASP was passed over a sensor chip with immobilized GST-C/RWB as well as GST-WIPWB (Fig. 2*A*, *bottom*). Both curves were fitted to a 1:1 binding model. Myc-WASP was injected at varying concentrations (250, 125, 62.5, 37.5, 18.8, and 9.38 nM) onto the chip, and dissociation constants (K_D) of Myc-WASP for GST-WIPWB and GST-C/RWB were determined from these results. The K_D values were 1.02×10^{-10} and 9.52×10^{-9} M for GST-WIPWB and GST-C/RWB, respectively. These results indicate that both WIP and WICH/WIRE bind directly to WASP and that there is no significant difference in the affinity of WASP for the either of two mammalian verprolins, WIP or WICH/WIRE.

THP-1 cells were examined to determine whether WASP binds to WIP or WICH/WIRE in cells, because THP-1 cells express each WASP, WIP, and WICH/WIRE (Fig. 1*D*). WASP was immunoprecipitated with anti-WASP mAb from THP-1 cell lysates (Fig. 2*B*, *lane 2*). Both WIP and WICH/WIRE were detected in the anti-WASP immunoprecipitates (Fig. 2*B*, *lanes 4* and *6*), indicating that WASP binds both WIP and WICH/WIRE in THP-1 cells.

The role of WASP and mammalian verprolins in monocyte chemotaxis

To determine whether WASP and mammalian verprolins (WIP and WICH/WIRE) play a role in monocyte chemotaxis, expression of WASP or mammalian verprolins was silenced in THP-1 cells by transfection of siRNA, and then chemotactic migration of THP-1 cells in response to a chemokine, MCP-1, was assayed. When THP-1 cells were transfected with siRNAs for WASP, WIP, and WICH/WIRE, expression of mRNAs encoding these proteins was analyzed by RT-PCR. siRNA transfection reduced expression of these three mRNAs (Fig. 3*A*). Levels of these three proteins were also examined by immunoblotting. siRNA transfection decreased the amount of all three proteins in THP-1 cells (Fig. 3*B*). We then examined chemotactic migration of THP-1 cells in response to MCP-1, when expression of those proteins was silenced. When WASP expression was silenced by WASP siRNA (siWASP), THP-1 chemotaxis was reduced (Fig. 3*C*), indicating that WASP plays a critical role in monocyte chemotaxis. This result is consistent with the observation that chemotactic migration of monocytes were impaired in WASP-deficient WAS patients (34, 35, 37, 38, 40–43). When expression of both WIP and WICH/WIRE was silenced by WIP and WICH/WIRE siRNA (siWIP and siC/R), THP-1 chemotaxis was also reduced (Fig. 3*C*). This result indicates that WIP and WICH/WIRE also play an important role in monocyte chemotaxis. This is the first result indicating that mammalian verprolins are involved in chemotaxis. Introduction of scrambled control siRNAs for WASP (sc(A)), WIP (sc(I)), and WICH/WIRE (sc(C/R)) barely affected chemotactic migration of THP-1 cells, compared with migration of untransfected THP-1 cells (control, □).

The role of the WASP-verprolin complex in monocyte chemotaxis

We next asked whether formation of a complex of WASP with mammalian verprolin is critical for chemotaxis by blocking binding of WASP to mammalian verprolins in THP-1 cells and assaying those cells for chemotactic migration.

To do so, EGFP-tagged WIP C-terminal fragment containing the WASP binding domain of WIP (residues 321–503) (EGFP-WB) was expressed in THP-1 cells. The WASP binding domain of WIP was predicted to block WASP binding to mammalian verprolins, because the WIP C-terminal fragment (residues 321–503) binds directly to WASP (Fig. 2*A*) and both WIP and WICH/WIRE bind to the same site of WASP (23–25). After transfection of THP-1 cells with the EGFP-WB expression construct, EGFP-positive cells expressing EGFP-WB were sorted by FACS. As a control,

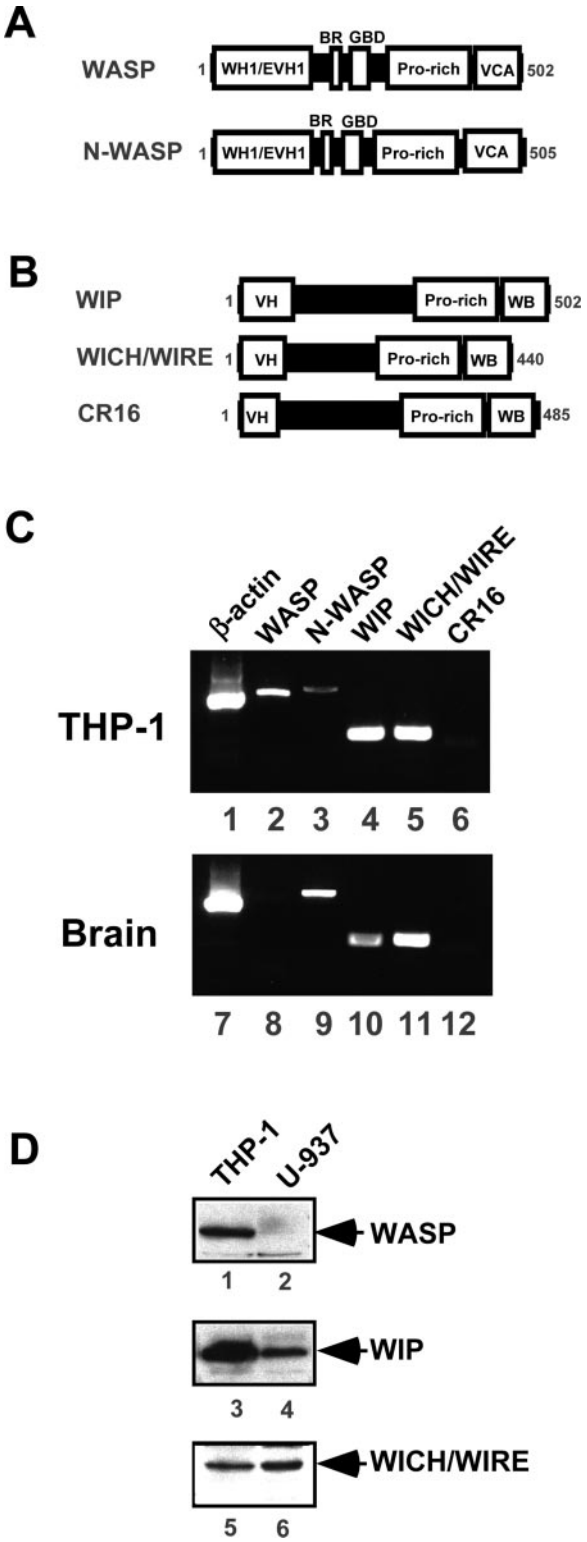

FIGURE 1. Expression of WASP family proteins and mammalian verprolins in monocytes. *A*, Domain organization of WASP and N-WASP. WH1, WASP homology 1; EVH1, Ena/vasodilator stimulated phosphoprotein (VASP) homology 1; BR, basic protein region; Pro-rich, proline-rich region; VCA, verprolin/cofilin/acidic domain. *B*, Domain organization of mammalian homologs of verprolin. V_H, Verprolin homology; WB, WASP-binding domain. *C*, Expression of two WASP family proteins and three mammalian verprolins were analyzed by RT-PCR. RT-PCR was performed with total RNA obtained from THP-1 cells (*lanes 1–6*) and human brain (*lanes 7–12*). Primer sets for β-actin, WASP, N-WASP, WIP, WICH/WIRE, and CR16 provided 661, 710, 830, 275, 289, and 250 bp of amplified fragments, respectively. Fragments were analyzed on a 1% agarose gel. *D*, Expression of WASP, WIP, and WICH/WIRE was analyzed by immunoblotting. One hundred micrograms of total protein prepared from two human monocyte cell lines, THP-1 (*lanes 1*, *3*, and *5*) and U-937 (*lanes 2*, *4*, and *6*), were subjected to SDS-PAGE followed by immunoblotting with anti-WASP polyclonal Ab (*lanes 1* and *2*), anti-WIP polyclonal Ab (*lanes 3* and *4*), and anti-WICH/WIRE polyclonal Ab (*lanes 5* and *6*).

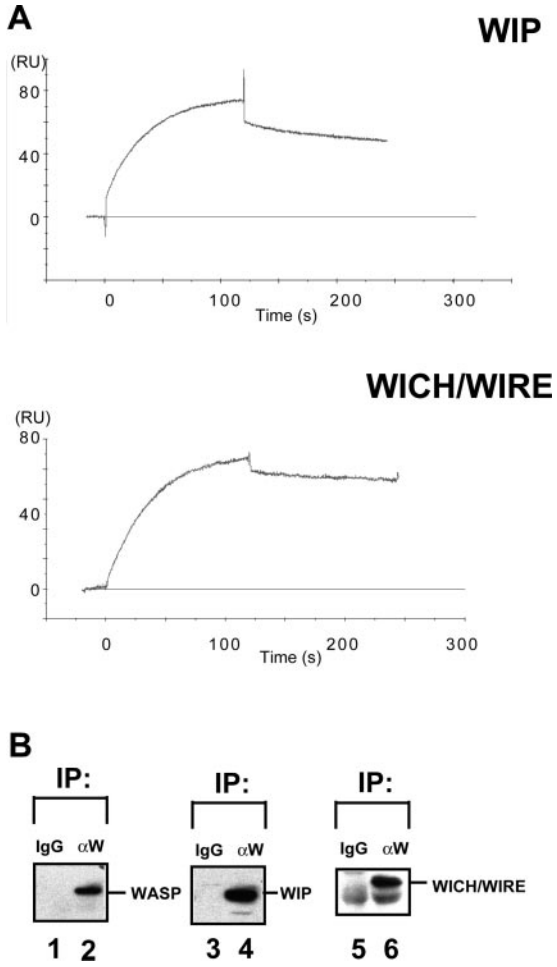

FIGURE 2. Characterization of WASP binding to mammalian verprolins. *A*, In vitro analysis of direct binding of WASP to mammalian verprolins by Biacore 3000. GST-fusion proteins of WASP binding domains of WIP or WICH/WIRE (GST-WIPWB or GST-C/RWB) were purified and immobilized onto the sensor chip. Myc-tagged WASP (full-length, Myc-WASP) was injected onto the chip at varying concentrations (250, 125, 62.5, 37.5, 18.8, and 9.38 nM). Sensorgrams obtained from the experiments in which Myc-WASP was injected at 250 nM are shown. GST-WIPWB (*top*) and GST-WICH/WIRE (*bottom*) were immobilized on the chip. *B*, Binding of WASP to mammalian verprolins in THP-1 cells. WASP was immunoprecipitated from the lysate of human monocyte cells, THP-1 followed by immunoblotting with anti-WASP polyclonal Ab (*lanes 1* and *2*), anti-WIP polyclonal Ab (*lanes 3* and *4*), and anti-WICH/WIRE polyclonal Ab (*lanes 5* and *6*).

THP-1 cells were transfected with pEGFP-C2 only, and cells expressing EGFP only were also sorted. WASP, WIP, and WICH/WIRE were expressed at the same levels in THP-1 transfected cells expressing either EGFP or EGFP-WB (Fig. 4*A*, *lanes 1–8*). WASP was immunoprecipitated with an anti-WASP mAb from these two THP-1 transfected cells (Fig. 4*A*, *lanes 9* and *10*). When EGFP-WB was expressed in THP-1 cells, EGFP-WB coimmunoprecipitated with WASP, indicating that EGFP-WB bound to

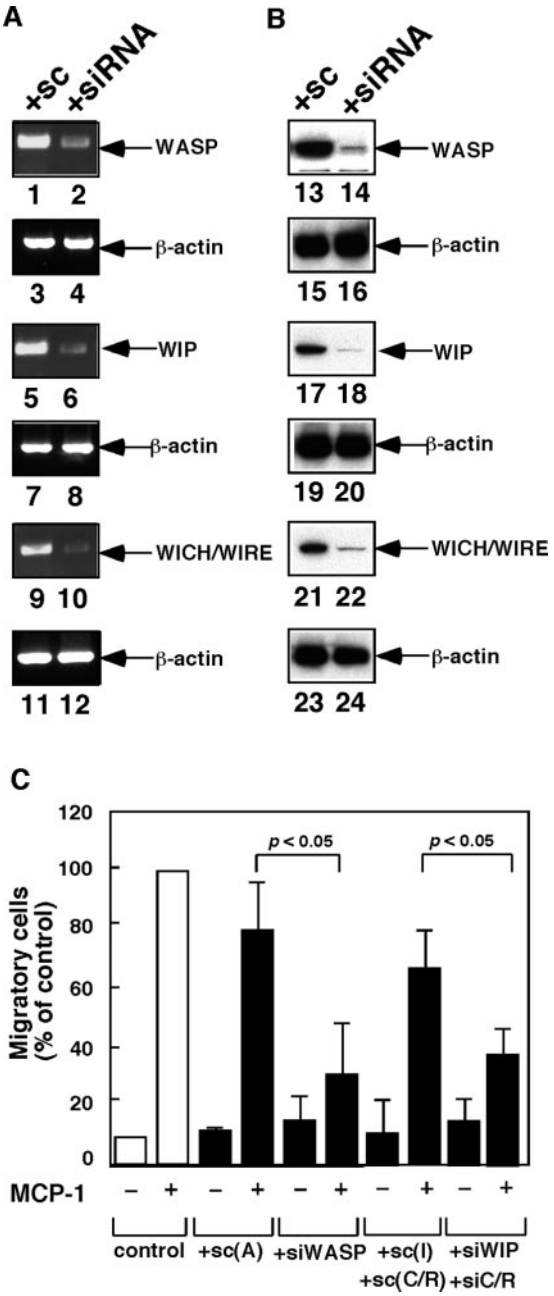

FIGURE 3. WASP and mammalian verprolins play a role in monocyte chemotaxis. *A*, *B*, Expression of WASP, WIP, and WICH/WIRE was reduced by transfection of siRNA. THP-1 cells were transfected with siRNA for WASP (*lanes 2*, *4*, *14*, and *16*) or a scrambled control (*lanes 1*, *3*, *13*, and *15*). Cells were cotransfected with siRNAs for WIP and WICH/WIRE (*lanes 6, 8, 10, 12, 18, 20, 22, 24*) or scrambled controls (*lanes 5*, *7*, *9*, *11*, *17*, *19*, *21*, and *23*). *A*, Total RNA was prepared from the transfectants, and transcription of each gene was analyzed by RT-PCR. *B*, Total protein was prepared from the transfectants and protein expression level was examined by immunoblotting. *C*, MCP-1-dependent chemotaxis of THP-1 cells was measured. □, Chemotaxis of untransfected THP-1 cells. ■, Chemotaxis of transfected THP-1 cells. siWASP and sc(A) indicate siRNA for WASP and a scrambled control, respectively. siWIP, siC/R, sc(I), and sc(C/R) indicate siRNA for WIP, siRNA for WICH/WIRE, a scrambled control for siWIP, and a scrambled control for siC/R, respectively. Data represent the mean ± SD of triplicate measurements.

WASP in the THP-1 cells (Fig. 4*A*, *lane 16*). The amount of coimmunoprecipitated WIP from THP-1 cells expressing EGFP-WB was much lower than that from THP-1 cells expressing EGFP only (Fig. 4*A*, *lanes 11* and *12*). The amount of coimmunoprecipitated WICH/WIRE from EGFP-WB-expressing THP-1 cells was also lower than from EGFP-expressing cells (Fig. 4*A*, *lane 13* and *14*). These results indicate that EGFP-WB bound to WASP, blocking binding of WASP to both WIP and WICH/WIRE in the THP-1 cells. We thus blocked binding of WASP to both mammalian verprolins, WIP and WICH/WIRE, without affecting expression of either protein (Fig. 4*A*).

Chemotactic migration of THP-1 cells expressing EGFP-WB was measured and compared with THP-1 cells expressing EGFP only. There is no significant difference in chemotactic migration stimulated by MCP-1 between control (untransfected) cells (control, □) and cells expressing EGFP only (Fig. 4*B*), indicating that chemotaxis is little affected by transfection or EGFP expression. However, THP-1 cells expressing EGFP-WB exhibited significantly reduced chemotactic migration in response to MCP-1 (Fig. 4*B*), indicating that WASP binding to verprolins was critical for chemotaxis.

To confirm that a complex of WASP with verprolins plays an important role in monocyte chemotaxis, we transfected the U-937 monocyte cell line, which do not express WASP and expresses very low levels of WIP (Fig. 1*D*), with WASP and WIP expression constructs. U-937 cells were cotransfected with WASP, WIP, or pEGFP-C2, the latter as a transfection control. Two days after transfection, EGFP-positive U-937 cells were sorted by FACS and analyzed. Expression of both WASP and WIP was increased in U-937 transfected cells (Fig. 5*A*, *lanes 1–4*). WIP coimmunoprecipitated with WASP, indicating that WASP binds to WIP in transfected U-937 cells (Fig. 5*A*, *lanes 5* and *6*).

Because U-937 cells do not express the MCP-1 receptor and therefore do not respond to MCP-1, chemotactic migration of U-937 cells was assayed in response to SDF-1α. There was no significant difference in chemotactic migration to SDF-1α between control U-937 (untransfected) and mock-transfected U-937 cells (Fig. 5*B*), indicating that transfection has negligible effect on U-937 chemotaxis. Transfected cells showed ~50% increases in chemotactic migration compared with mock-transfected cells (Fig. 5*B*), indicating that when WASP and WIP are coexpressed in U-937 cells and form a complex, chemotactic migration in response to SDF-1α is significantly increased. This result, together with the results in Figs. 3 and 4, strongly suggests that a complex of WASP with WIP plays an important role in monocyte chemotaxis.

Actin polymerization and polarization of THP-1 cells

We next examined actin polymerization and cell polarization, because both processes are prerequisite to and essential for chemotaxis. We measured actin polymerization and examined cell polarization of THP-1 cells upon MCP-1 stimulation. When THP-1 cells were transfected with siRNA for WASP, F-actin content was reduced and cell polarization was impaired, compared with when transfected with scrambled control siRNA (scWASP) (Fig. 6, *A*, *B*, and *G*). This is consistent with the phenotype of monocytes from WAS patients (39, 42). This result confirmed that WASP plays an important role in actin polymerization and cell polarization in monocytes.

When THP-1 cells were transfected with siRNAs for WIP and WICH/WIRE, F-actin content was also reduced and cell polarization was impaired, compared with cells transfected with control siRNAs (scWIP and scC/R) (Fig. 6, *C*, *D*, and *G*). This result suggests that mammalian verprolins also play an important role in actin polymerization and cell polarization in monocytes.

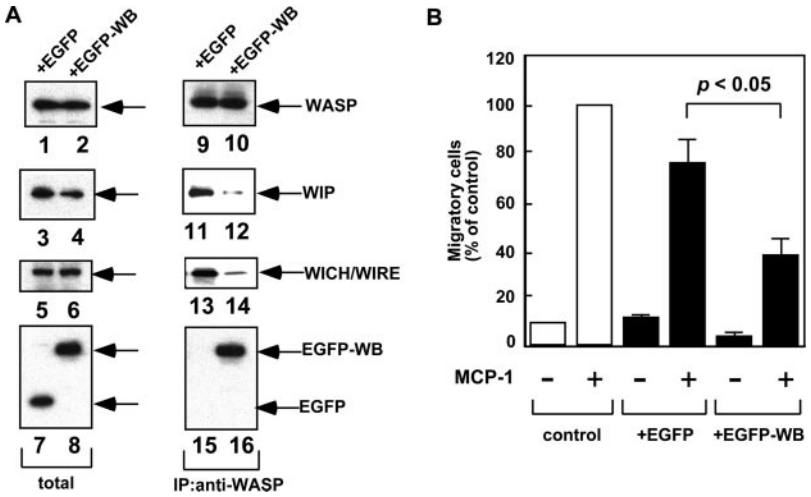

FIGURE 4. Binding of WASP to WIP and WICH/WIRE is important for monocyte chemotaxis. *A*, Binding of WASP to WIP and WICH/WIRE was blocked by EGFP-WB. THP-1 cells were transfected with pEGFP-C2 alone (*lanes 1*, *3*, *5*, *7*, *9*, *11*, *13*, and *15*) or the EGFP-tagged WASP binding site of WIP (residues 321–503) (EGFP-WB) (*lanes 2*, *4*, *6*, *8*, *10*, *12*, and *14*). Total lysates were analyzed by immunoblotting (*lanes 1–8*) using anti-WASP (*lanes 1* and *2*), anti-WIP (*lanes 3* and *4*), anti-WICH/WIRE (*lanes 5* and *6*), and anti-EGFP (*lanes 7* and *8*). WASP was immunoprecipitated with anti-WASP (*lanes 9–16*) followed by immunoblotting using anti-WASP (*lanes 9* and *10*), anti-WIP (*lanes 11* and *12*), anti-WICH/WIRE (*lanes 13* and *14*) and anti-EGFP (*lanes 15* and *16*). *B*, Chemotactic migration of THP-1 cells in response to MCP-1. Chemotactic migration of THP-1 cells untransfected (□) and transfected with EGFP or EGFP-WB (■). +EGFP, THP-1 cells transfected with pEGFP-C2 alone; +EGFP-WB, THP-1 cells transfected with the EGFP-tagged WASP binding site of WIP (residues 321–503). Data represent the mean ± SD of triplicate measurements.

We then asked whether binding of WASP to verprolins is critical for actin polymerization and cell polarization. To block binding of WASP to verprolins, THP-1 cells were transfected with HcRed constructs (pHcRed alone or pHcRed-WB) for actin polymerization and EGFP constructs to analyze cell polarization. Two days after transfection, HcRed-positive THP-1 cells were sorted by FACS and stimulated with MCP-1. Cells were fixed, permeabilized, and stained with Alexa 488-phalloidin to measure F-actin content. Cell polarization was examined by phase-contrast microscopy. When binding of WASP to WIP was blocked by the WASP binding domain (HcRed-WB) in THP-1 cells, actin polymerization occurred, although it was mildly affected (Fig. 6, *E* and *F*), but cell polarization was significantly impaired (Fig. 6*G*). Thus, when the complex formation of WASP with mammalian verprolins was inhibited, cells still can polymerize actin but do not establish polarization. Impaired polarization results in reduced chemotactic migration (Fig. 4*B*).

WASP-verprolin complex in primary human monocytes

To validate the results obtained from experiments using cell lines, it is necessary to extend the study from cell lines to primary cells. We examined freshly isolated human monocytes if the WASP-verprolin complex is involved in chemotaxis.

We isolated human monocytes from freshly drawn peripheral blood and transfected them with EGFP, EGFP-WB, HcRed, or HcRed-WB constructs using amaxa nucleofector II. The transfection efficiency was estimated to be ~50–60% based on the expression of EGFP analyzed by a flow cytometer. We used the transfected cells for the experiments without concentrating by a cell sorter. When WASP binding to verprolins was blocked by the expression of EGFP-WB, chemotactic migration of monocytes in response to MCP-1 was significantly decreased (Fig. 7*A*). Blocking binding had no effect on actin polymerization (Fig. 7*B*) but significantly reduced cell polarization of monocytes (Fig. 7*C*).

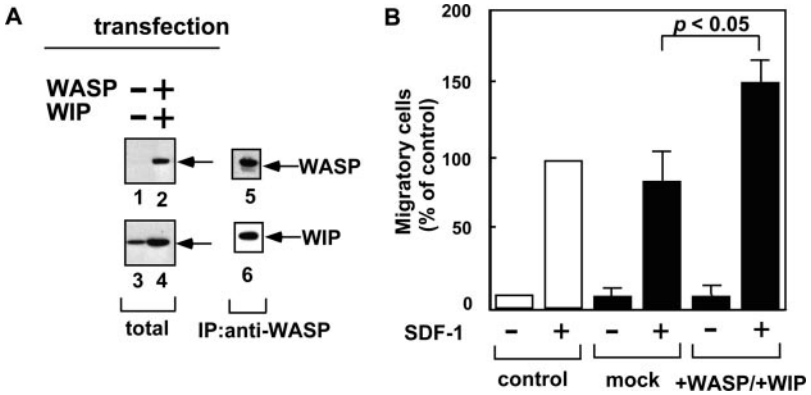

FIGURE 5. Binding of WASP to mammalian verprolin is required for monocyte chemotaxis. *A*, U-937 cells were cotransfected with WASP, WIP, and pEGFP. After transfection, EGFP-positive cells were sorted by FACSDiva. Sorted cells were used for a chemotaxis assay and immunoblotting. Total lysates were analyzed by immunoblotting using anti-WASP (*lanes 1* and *2*) and anti-WIP (*lanes 3* and *4*) Abs. WASP was immunoprecipitated with anti-WASP mAb from U-937 cells transfected with WASP and WIP followed by immunoblotting using anti-WASP (*lane 5*) and anti-WIP (*lane 6*) Abs. *B*, Chemotactic migration of U-937 cells in response to SDF-1α was measured. Chemotactic migration of untransfected U-937 cells (□) and mock-transfected U-937 cells and cells transfected with WASP and WIP (■). Data represent the mean ± SD of triplicate measurements.

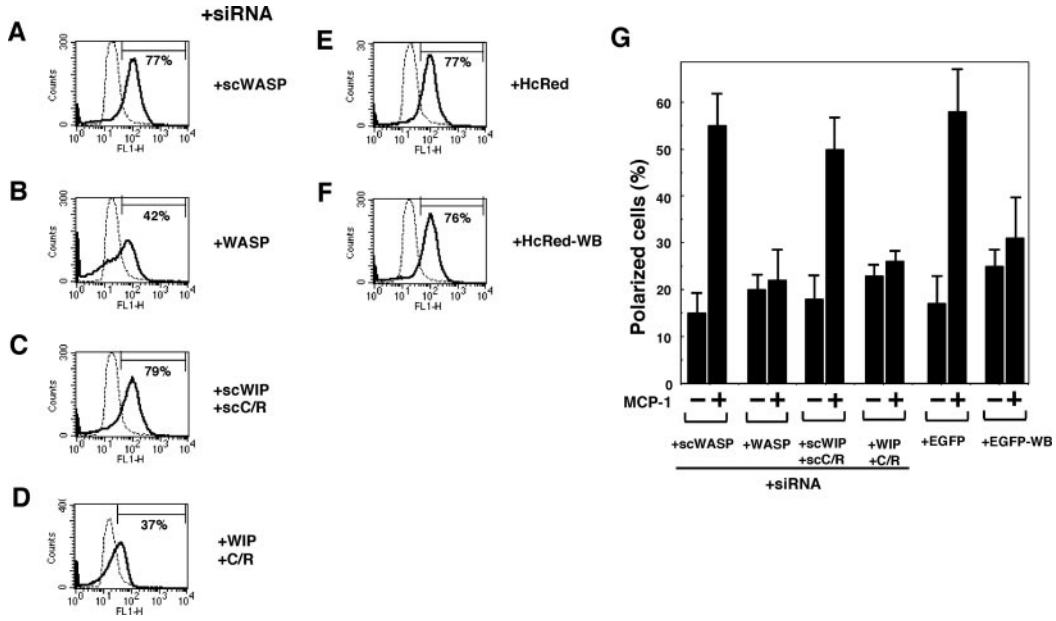

FIGURE 6. MCP-1-dependent actin polymerization and cell polarization of THP-1 cells. *A–F*, THP-1 cells were transfected with the scrambled control for WASP (*A*), siRNA for WASP (*B*), scrambled controls for WIP and WICH/WIRE (*C*), siRNAs for WIP and WICH/WIRE (*D*), and HcRed constructs (pHcRed alone and pHcRed-WB) (*E* and *F*). Two days after transfection, HcRed-positive cells were sorted and stimulated with 10 nM MCP-1 for 15–30 min. Cells were then fixed, permeabilized, and stained with Alexa 488-phalloidin. Cells were analyzed for F-actin content by FACS. Dotted histograms and bold-line histograms indicate unstimulated cells and cells stimulated with MCP-1, respectively. *B*, THP-1 cells were transfected with siRNAs or EGFP constructs. Two days after transfection, EGFP-positive cells were sorted and stimulated with 10 nM MCP-1 for 15–30 min, examined under phase-contrast microscopy, and visually scored. Data represent the mean ± SD of triplicate measurements.

These results are essentially identical with the results obtained from the experiments using THP-1 (Figs. 4 and 6), validating that the WASP-verprolin complex plays a critical role in human monocytes.

The role of the WASP-verprolin complex in podosome formation and phagocytosis

We next determined whether WASP binding to verprolins is involved in other monocyte functional activities such as formation of podosome, a cell adhesion structure, and phagocytosis, because WASP is critical for these cellular processes (49–52).

Macrophages form actin-rich adhesion structures called podosomes. Macrophages from WASP-deficient WAS patients completely lacked podosomes (49), and expression of exogenous WASP restored the formation of podosomes (42). To analyze podosome formation, THP-1 cells were differentiated by stimulation with PMA to obtain a macrophage-like phenotype 1 day before transfection, which closely resembles human monocyte-derived

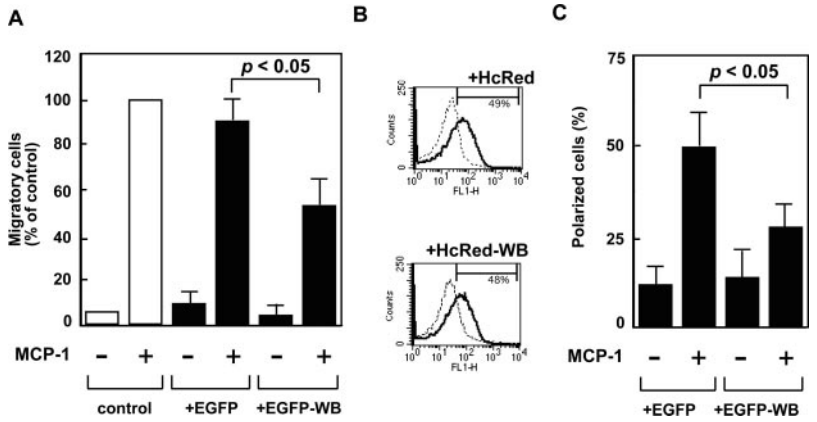

FIGURE 7. WASP binding to verprolins is important for chemotaxis, cell polarization of human primary monocytes. *A*, Chemotactic migration of human primary monocytes in response to MCP-1. Freshly isolated human primary monocytes were transfected with EGFP or EGFP-WB constructs using amaxa nucleofector. Chemotactic migration of untransfected cells (□) and cells transfected with EGFP or EGFP-WB (■) were assayed. +EGFP, Cells transfected with pEGFP-C2 alone; +EGFP-WB, cells transfected with the EGFP-tagged WASP binding site of WIP (residues 321–503). *B*, MCP-1-dependent actin polymerization of human primary monocytes. Cells were transfected with HcRed or HcRed-WB constructs. Cells were then fixed, permeabilized, and stained with Alexa 488-phalloidin. Cells were analyzed for F-actin content by FACS. Dotted histograms and bold-line histograms indicate unstimulated cells and cells stimulated with MCP-1, respectively. *C*, MCP-1-dependent cell polarization of human primary monocytes. Cells were transfected with EGFP or EGFP-WB constructs using amaxa nucleofector. Cell polarization of untransfected cells (□) and cells transfected with EGFP or EGFP-WB (■) were assayed. +EGFP, Cells transfected with pEGFP-C2 alone; +EGFP-WB, cells transfected with the EGFP-tagged WASP binding site of WIP (residues 321–503). Data represent the mean ± SD of triplicate measurements.

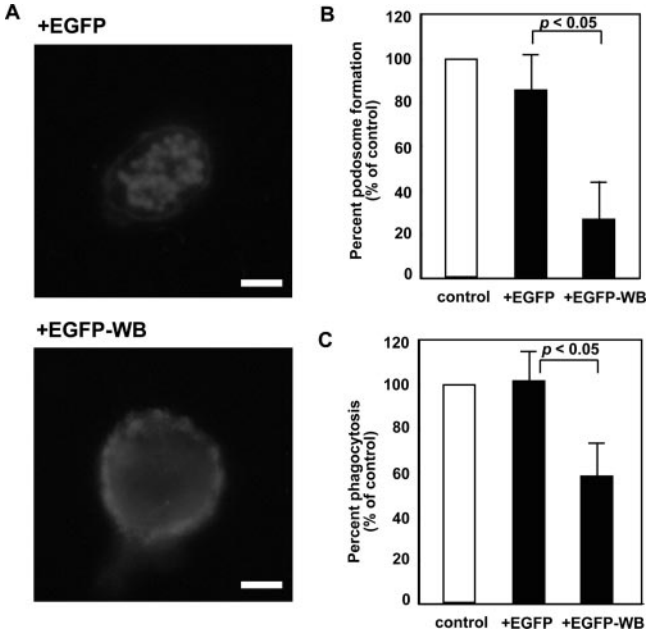

FIGURE 8. Binding of WASP to verprolins is involved in podosome formation and phagocytosis. *A*, Podosome formation in PMA-differentiated THP-1 cells stained with Alexa 568-phalloidin. Differentiated THP-1 cells were transfected with EGFP (*top*) and EGFP-WB (*bottom*) to block binding. Bar is 20 μm. *B*, Podosome formation in differentiated THP-1 cells. Shown is the percentage of cells forming podosomes per total cells counted (200 cells). *C*, Phagocytosis of differentiated THP-1 cells. Uptake of fluorescent latex beads into cells was assayed. Shown is the percentage of cells phagocytosing at least one bead per total cells counted (200 cells).

macrophages, as previously reported (53, 54). A day later, cells were transfected with EGFP or EGFP-WB. Two days after transfection, cells were stained with Alexa 568-phalloidin. Only EGFP-positive cells were examined for podosome formation. Actin cores of podosomes stained with phalloidin gave a punctate staining pattern (Fig. 8*A*, *top*). The number of cells forming and not forming podosomes was counted under a fluorescence microscope. The percentage of cells forming podosomes per total cells counted are shown in Fig. 8*B*. When WASP binding to verprolins was blocked by EGFP-WB, podosome formation was significantly impaired (Fig. 8, *A*, *bottom*, and *B*).

Next, we asked whether blocking WASP binding to verprolins affects phagocytosis. PMA-differentiated THP-1 cells were incubated with serum-opsonized fluorescence-labeled latex beads for in vitro phagocytosis, and the uptake of beads was examined. When WASP binding to verprolins was blocked by EGFP-WB, the percent phagocytosis was decreased to ~60% of control (Fig. 8*C*). Blocking WASP binding to verprolins significantly attenuated phagocytosis.

Collectively, these results indicate that, in addition to chemotaxis, the WASP-verprolin complex is involved in both podosome formation and phagocytosis.

Discussion

In the present study, we show that WASP-binding proteins, the mammalian verprolins, play an important role in monocyte chemotaxis (Fig. 3). Furthermore, we demonstrated that complex formation of WASP with verprolins is important for monocyte chemotaxis (Figs. 4, 5, and 7). When binding of WASP to verprolins was blocked, actin polymerization occurred, but cell polarization was impaired (Fig. 6). Our results suggest that WASP and WIP function as a unit to establish cell polarization required for monocyte chemotaxis, which is supported by the finding that the yeast WASP homolog, Las17/Bee1 interacts with verprolin (Vrp1/End5) and that the interaction is essential for the polarized cortical actin cytoskeleton and endocytosis (55).

When binding of WASP to verprolins was blocked, the activity of the WASP-verprolin complex required to establish cell polarization was reduced (Fig. 6*B*). Alternatively, when binding was blocked, WASP localization is likely altered, because WIP is crucial to localize WASP activity (28, 29). Actin polymerization occurs but does not occur at appropriate sites in monocytes due to altered WASP localization, resulting in impaired cell polarization (Fig. 6*B*).

Our results also suggest a possible disease mechanism underlying recurrent infection in WAS patients. The complex of WASP with verprolins can stimulate actin polymerization at appropriate sites, and then establish cell polarization. In WASP-deficient WAS patients, the complex is not formed and the activity of WASP-verprolin unit is lost due to the absence of WASP, resulting in impaired chemotactic migration. Impaired migration causes defects in monocyte and leukocyte recruitment to inflamed tissues, resulting in recurrent infections in WAS patients.

In WIP-deficient mice, T cells fail to proliferate, secrete IL-2, increase their F-actin content, and polarize in response to TCR ligation, and they form smaller T cell-APC conjugate interfaces (58). In mast cells from WIP-deficient mice, IgE-receptor-mediated cell activation is impaired (59). In addition, in WASP and WIP double-knockout mice, T cell homing and chemotaxis are partially reduced (60). However, chemotactic defects in monocytes/macrophages were not shown in these studies, suggesting that mice have a backup mechanism for monocyte chemotaxis that may include other family members such as N-WASP and WICH/WIRE. Residual N-WASP and WICH/WIRE activity in these model mice might make detection of chemotactic defects in monocytes/macrophages difficult to analyze. We showed that WASP, WIP, and WICH/WIRE are expressed predominantly in THP-1 cells (Fig. 1*C*), and binding of WASP to WIP and WICH/WIRE was blocked in THP-1 cells (Fig. 4*A*). Thus, we could examine the effect of blocking binding of WASP to both verprolins on chemotaxis and detect chemotactic defects in monocytes. Further studies are needed to analyze the potential role of complex formation of WASP with verprolins in other chemotactic processes such as transendothelial chemotactic migration.

Blocking WASP binding to verprolins barely affects actin polymerization itself (Fig. 6, *E* and *F*) but impaired podosome formation (Fig. 8, *A* and *B*). These results suggest that the WASP-verprolin complex is required for concentrating F-actin at an appropriate site to form podosomes.

Patients from X-linked thrombocytopenia (XLT), a milder form of WAS, have missense mutations in the WASP N terminus (residues 1–137), which is required for binding to verprolins (3, 23). XLT patients express the mutant proteins at a lower concentration than normal subjects (3, 4, 61, 62), and defects were observed in only platelets, but not in other hemopoietic cells (3, 4). Recently, Linder et al. (57) reported that XLT macrophages, previously thought to be unaffected in this disorder, are compromised in podosome formation. Complex formation of mutant WASPs with verprolins is most likely impaired in XLT patients, because XLT mutations reduced WASP binding to WIP (56). These studies suggest that the inefficient formation of the WASP-verprolin complex causes reduced podosome formation. Our results (Fig. 8, *A* and *B*) are consistent with these studies.

Phagocytic defect causes impaired clearance of apoptotic cells, allowing the apoptotic cells to become available to resident dendritic cells. Those dendritic cells present self-Ags to the immune

system in an inflammatory context, resulting in autoimmunity (63). Phagocytic defect is thus thought to be a cause of autoimmunity. Blocking WASP binding to verprolins attenuated phagocytosis (Fig. 8*C*). Phagocytic defect was observed in macrophages from WASP-deficient WAS patients (50–52). These results suggest that the WASP-verprolin complex plays a role in phagocytosis. Autoimmunity in WAS patients may be caused by phagocytic defect due to inefficient formation of the WASP-verprolin complex.

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Disclosures

The authors have no financial conflict of interest.

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