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Identification of Two Human WAVE/SCAR Homologues as General Actin Regulatory Molecules Which Associate with the Arp2/3 Complex

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WAVE/SCAR protein was identified as a protein which has similarity to WASP and N-WASP, especially in its C terminal. Recently, WAVE/SCAR protein has been shown to cooperate with the Arp2/3 complex, a nucleation core for actin polymerization *in vitro*. However, in spite of its general function, WAVE/SCAR expression is mainly restricted to the brain, suggesting the existence of related molecule(s). We here identified two human WAVE/SCAR homologues, which cover other organs. We named the original WAVE1 and newly identified ones WAVE2 and WAVE3. WAVE2 had a very wide distribution with strong expression in peripheral blood leukocytes and mapped on chromosome Xp11.21, next to the WASP locus. WAVE3 and WAVE1 had similar distributions. WAVE3 was strongly expressed in brain and mapped on chromosome 13q12. WAVE1 was mapped on chromosome 6q21-22. Ectopically expressed WAVE2 and WAVE3 induced actin filament clusters in a similar manner with WAVE1. These actin cluster formations were suppressed by deletion of their C-terminal VPH (verproline homology)/WH2 (WASP homology 2) domain. Further, WAVE2 and WAVE3 associate with the Arp2/3 complex as does WAVE1. Our identification of WAVE homologues suggests that WAVE family proteins have general function for regulating the actin cytoskeleton in many tissues.

Actin is a primary determinant of cell shape and motility. In response to various signals, actin cytoskeleton is dynamically reorganized (1, 2). However, we do not know how actin cytoskeleton is reorganized at molecular level. In the process of actin cytoskeletal reorganization, actin filaments are depolymerized and then actin monomers polymerize. In this point, WASP family proteins such as WASP, N-WASP, and WAVE/SCAR have interesting characteristics, because WASP family proteins depolymerize actin filament by themselves and enhance actin polymerization by forming complex with other actin regulatory molecules such as Arp2/3 complex and profilin (3–12).

We first identified WAVE as a protein which has similarity to WASP and its relative, N-WASP, by DNA sequence database search as KIAA0269 (8). In the same period, the same protein was also identified as SCAR, which is involved in tip formation and chemotaxis in *Dictyostelium* (9). In addition, profilin and small GTPase Rac are shown to have functional involvements with WAVE/SCAR (8). Recent studies demonstrated that WAVE/SCAR cooperates with an actin nucleation core Arp2/3 complex in enhancing actin polymerization *in vitro* as well as N-WASP does (10–12). These characteristics of WAVE suggest that WAVE functions as an important executor which directly links upstream signals to actin cytoskeletal reorganizations.

In spite of its general function of WAVE, its distribution is strictly limited to brain (13). This fact prompted us to search WAVE homologues expressed in other tissues. Here, by database search and library screening, we identified and mapped two WAVE/SCAR homologues named WAVE2 and WAVE3. We named original WAVE/SCAR WAVE1. In contrast to WAVE1, WAVE2 was ubiquitously expressed in all tissues except in skeletal muscle cells. WAVE3 also had restricted distribution in brain. All WAVE proteins seemed to function in a similar manner through association with Arp2/3 complex at C-termini and with actin at their VPH domains (8, 14). These findings suggest that WAVE family proteins are essential and ubiquitous factor for regulating actin cytoskeleton.

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Abbreviations used: aa, amino acid(s); SHD, scar homology domain; WASP, Wiskott–Aldrich syndrome protein; VPH, verproline homology; WH2, WASP homology 2; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bp, base pairs.

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MATERIALS AND METHODS

Cloning of WAVE/SCAR homologues. The EST clones and genomic clones which have homology to WAVE1 were identified by database search and roughly categorized. The plasmids bearing EST clones which might consist of new groups of WAVE such as GenBank accession ID: aa452030, aa062862, aa567420, aa708499, w40757, h38554, h18214, aa535513, aa445914, h50852, and d81963 were obtained from Genome Systems Inc. (St. Louis, MO) and other distributors. These plasmids were re-sequenced. Then, EST clones and genomic clones were further categorized. As a result, two close related genes to WAVE1 were suggested to exist. Using these EST clones and genomic clones as probes, Northern blotting and screening of cDNA libraries were performed. The probes used for WAVE2 and WAVE3 were amplified using PCR from genomic clone PAC393P12 (GenBank accession ID: AL022578) and EST clone aa452030, respectively. Probes for WAVE2 and WAVE3 correspond to nucleotides 42-397 of the WAVE2 cDNA and 48-439 of the WAVE3 cDNA, respectively. Several positive clones for WAVE2 and WAVE3 were obtained from cDNA libraries from Jurkat T cell lymphoma and human brain, respectively.

Northern blotting. Human multiple tissue Northern blots was purchased from CLONTECH. Hybridization was performed according to manufacture's instruction. Autoradiographies were performed using BAS 2000 Image Analyzer (Fuji Films). The probes for WAVE2 and WAVE3 were the same as those for library screening. Probe for WAVE1 corresponds to nucleotides 45-400. We confirmed that these probes do not cross-react with other WAVEs by Southern blotting against PCR templates (not shown).

Mapping on chromosomes. Chromosomal mapping was performed as described (15). The probes for WAVE1 and WAVE3 were 2- and 3-kb fragments from cDNA library, which contain all open reading frames, respectively.

GST-fusion proteins. Glutathione *S*-transferase (GST) fusion proteins of wild-type or ΔVPH-mutant (lacking amino acids 498–513, 437–452, and 441–456 for WAVE1, WAVE2, and WAVE3, respectively) C-terminal region of WAVEs (amino acids 436–559, 417–498, and 409–502 for WAVE1, WAVE2, and WAVE3, respectively) were constructed in pGEX plasmids (Pharmacia). These proteins were expressed in *Escherichia coli* and purified as described previously (8).

Binding assay against the Arp2/3 complex. Arp2/3 complex was purified as described (16). GST-fusion C-terminal WAVE proteins were immobilized on glutathione beads (Pharmacia), and mixed with purified Arp2/3 complex in buffer (10 mM imidazole (pH 7.0), 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM $CaCl_2$, 0.2 mM ATP, and 0.02% azide). After incubation at 4°C for 2 h, the beads were washed and bound proteins were analyzed by SDS–PAGE.

Binding assay against actin. GST-fusion C-terminal WAVE proteins were immobilized on glutathione beads and mixed with PC12 cell lysate suspended in buffer consisted of 40 mM Tris–HCl (7.5), 150 mM NaCl, and 0.5% Triton X-100. After washing, the bound proteins were analyzed by Western blotting using anti-actin antibody (Chemicon, Cat. No. MAB-1501).

Ectopic expression in COS-7 cells. Full-length FLAG-tagged wild type and ΔVPH (lacking amino acids 498–513, 437–452, and 441–456 for WAVE1, WAVE2, and WAVE3, respectively) mutant WAVEs-expression plasmids were constructed in pEF-BOS plasmid vector. FLAG-tag was attached to N-terminus of WAVEs. Then, ten micrograms of recombinant plasmid was transfected into COS-7 cells as described previously (8). After 30 h, the cells were fixed and harvested as described (8).

Immunofluorescence microscopy. Immunofluorescence microscopy was performed as follows. Cells fixed in 3.7% formaldehyde in PBS for 20 min were permeabilized with 0.2% Triton X-100 in PBS for 5 min. After incubation with anti-FLAG M2 antibody (Sigma) followed by anti-mouse antibody linked to fluorescein and rhodamine-conjugated phalloidin (Molecular Probes), the cells were observed by fluorescence microscopy.

RESULTS

Cloning of WAVE/SCAR homologues. In genome project, messenger RNAs have been intensively sequenced as expression sequence tags (ESTs). First, we performed EST database search to identify novel WAVE homologues because it seemed to be unusual that WAVE gene expression is restricted to brain in spite of its general function (13). Grouping of EST and genomic clones homologous to WAVE suggested that there were two genes that were very similar to WAVE. Using these EST and genomic clones as probes, we performed Northern blotting and screening of cDNA libraries, resulting in the identifications of two genes named WAVE2 (Genbank Accession: AB026542) and WAVE3 (AB026543) (Fig. 1A). We named original one WAVE1. These genes had stop codon just before its putative first methionine, and the sequence surrounding putative first methionine matched Kozak rule (17) (not shown). Thus, we concluded that these open reading frames encoded putative WAVE2 and WAVE3 proteins. WAVE2 is composed of 498 aa. WAVE3 is composed of 502 aa. Both WAVE2 and WAVE3 are smaller than WAVE1, which is composed of 559 aa. Comparison of the entire amino acid sequences showed that both WAVE2 and WAVE3 have 48% identity to WAVE1. Especially, the N- and C-terminal regions have high conservation, almost 80% identical (Fig. 1B).

Tissue distribution of WAVE1, WAVE2, and WAVE3. To analyze the tissue distribution of WAVE2 and WAVE3, we performed Northern blot analysis. In contrast to WAVE1, which is restricted to brain as described (13) (Fig. 2Aa), WAVE2 had a wide range of expression except in skeletal muscle. WAVE2 was expressed in brain, heart, colon, thymus, spleen, kidney, placenta, lung, and peripheral blood leukocyte (Fig. 2Ab). But its expression is not uniform. WAVE2 expression was very strong especially in placenta, lung, and peripheral blood leukocyte. Since WAVE1 and WAVE3 had little expression in thymus, spleen, and peripheral blood leukocytes, WAVE2 was suggested to have important functions in blood and immune system. In contrast to a wide distribution of WAVE2, WAVE3 gene expression was strong in brain as well as WAVE1. However, weak WAVE3 expression was observed in kidney and liver (Fig. 2Ac).

Chromosomal localization of WAVE1, WAVE2, and WAVE3. We performed fluorescent *in situ* hybridization to determine the chromosomal localization of WAVE1 and WAVE3. WAVE1 was mapped at chromosome 6q21-22. And WAVE3 was mapped on chromosome 13q12. WAVE2 chromosomal localization had al-

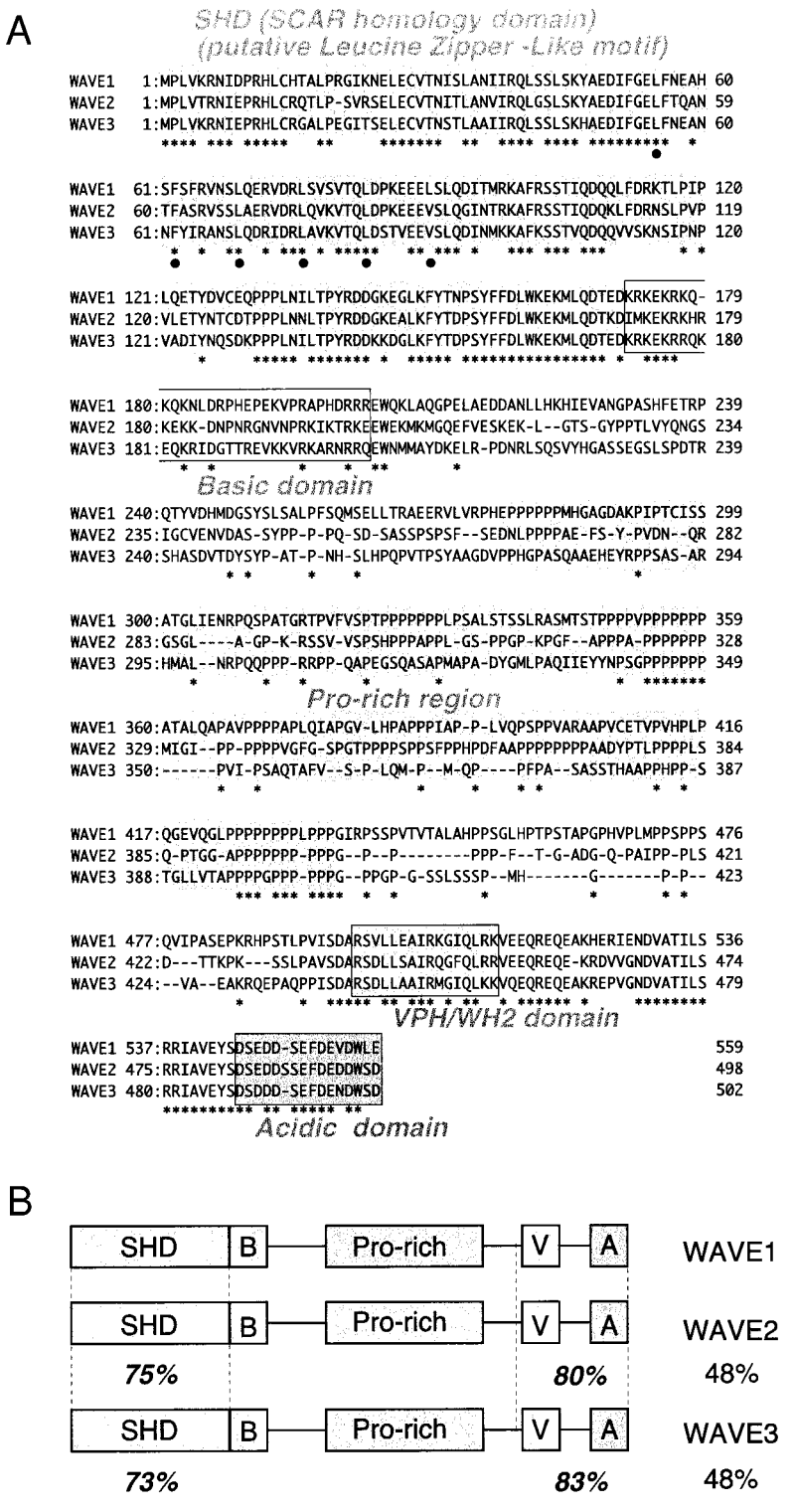
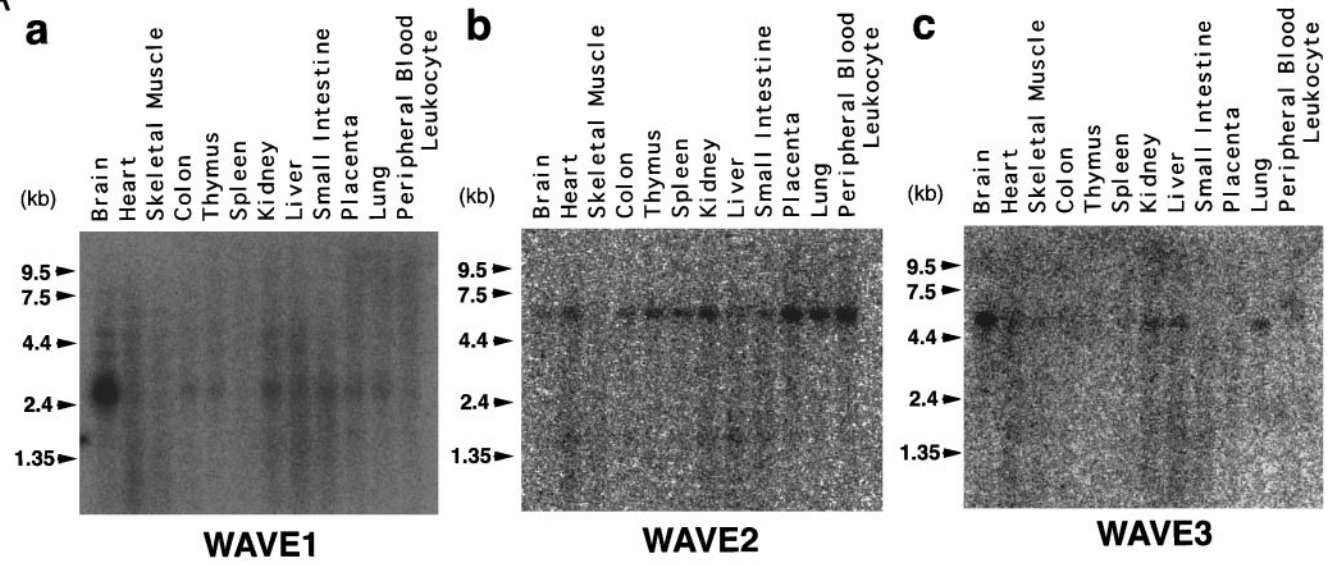


FIG. 1. Identification of WAVE2 and WAVE3. (A) Amino acid sequences of WAVE1 (Genbank Accession No. D87459 as KIAA0269), WAVE2 (AB026542) and WAVE3 (AB026543). Amino acid sequences were aligned using GENETIX Mac software and identical residues are shown as asterisks (*). Putative leucine zipper-like motif is shown as circles. Domain names are also shown. (B) Domain composition of WAVEs. The amino acid sequence identities of entire amino acids, SHD, and C-terminal region against WAVE1 were shown. B, V, and, A indicate basic domain, VPH domain, and acidic domain, respectively.

A



B

WAVE1	6q21-22	this study
WAVE2	Xp11.21	Bird, 1998 (PAC393P12 clone from Sanger Center, UK)
WAVE3	13q12	this study
WASP	Xp11.22	Derry et al, 1994
N-WASP	7q31.3	Fukuoka et al, 1997

FIG. 2. Tissue distribution of WAVEs. (A) Northern blot analysis of WAVEs. (a) WAVE1, (b) WAVE2, and (c) WAVE3. (B) Chromosomal localization of WASP family proteins such as WAVE1, WAVE2, WAVE3, WASP, and N-WASP. In this study, WAVE1 and WAVE3 were mapped by fluorescence *in situ* hybridization (FISH).

ready been determined by genome project at the chromosome Xp11.21, which is neighboring region of WASP gene at Xp11.22 (3) (Fig. 2B).

Association with the Arp2/3 complex and actin in the C-terminal region. Recent studies have demonstrated that both WAVE and N-WASP cooperate with Arp2/3 complex for enhancing the nucleation for actin polymerization *in vitro* (10–12). Both N-WASP and WAVE1 associate with Arp2/3 complex in its C-terminal region. Therefore, we examined the association between purified Arp2/3 complex and C-terminal region from WAVE1, WAVE2 or WAVE3 *in vitro.* The immobilized GST-fusion C-terminal WAVEs (Fig. 3A) and various amounts of purified Arp2/3 complex (Fig. 3B) were mixed together. After washing, the bound proteins were analyzed. As shown in Fig. 3C, all WAVE family proteins associate with Arp2/3 complex in a similar manner.

For WAVE1 to induce actin clusters, its verproline homology domain (VPH domain), a direct actin-binding domain, is shown to be essential (8). This VPH domain is well conserved in both WAVE2 and WAVE3. Therefore, we examined the association with actin through this VPH domain. As shown in Fig. 3D, all C-terminal regions of WAVEs associated with actin. Then, we deleted the VPH domain of WAVE2 and WAVE3. The ΔVPH mutants of C-terminal region of WAVEs did not associate with actin any more, indicating this domain is also necessary for WAVE2 and WAVE3 to bind to actin (Fig. 3D).

Ectopic expression in COS-7 cells. WAVE1 has been shown to induce abnormal actin clusters when overexpressed in cultured cells (8). To examine whether there is any difference in WAVE family, we expressed all WAVEs ectopically in COS-7 cells and observed their actin filaments with immunofluorescence microscopy.

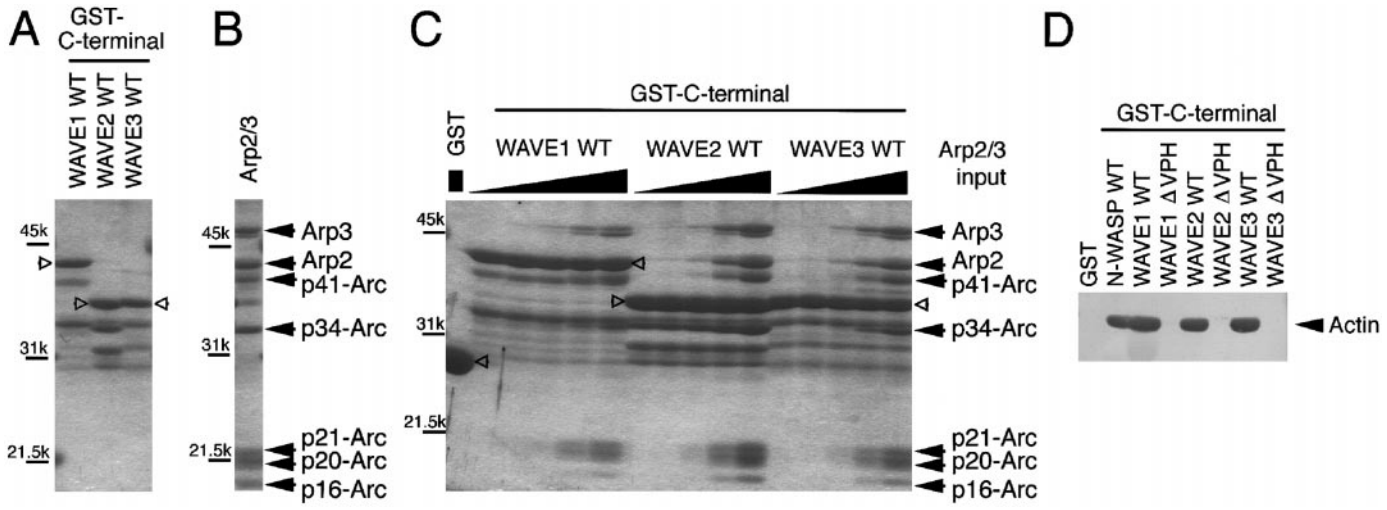


FIG. 3. Association with Arp2/3 complex and actin in its C-terminal region. (A) GST-C-terminal WAVEs used in this study. Purified GST-C-terminal WAVEs were analyzed by SDS-PAGE and Coomassie brilliant blue staining. White arrows indicate the purified proteins. Molecular weight markers are also shown as lines. (B) Purified Arp2/3 complex used in this study. Purified Arp2/3 complex was analyzed by SDS-PAGE and Coomassie brilliant blue staining. (C) Association with Arp2/3 complex. Purified Arp2/3 complex and immobilized GST-C-terminal WAVEs were mixed. Final concentrations of the Arp2/3 complex were 0.01, 0.04, 0.2, 0.8, and 4 μM. Association against GST was also examined as a control at 4 μM Arp2/3 complex concentration. After incubation and following washing, the binding proteins were analyzed by Coomassie brilliant blue staining. White arrows indicate GST-C-terminal WAVEs and arrows indicate the components of Arp2/3 complex. (D) Association with actin. PC12 cell lysates were mixed with immobilized wild type or ΔVPH mutant GST-C-terminal WAVEs and bound proteins were analyzed with Western blotting using anti-actin antibody.

As well as WAVE1, ectopic expression of WAVE2 and WAVE3 induced abnormal actin clusters that colocalized with expressed WAVEs (Fig. 4). Such actin cytoskeletal reorganizations were observed in most of the transfected cells we examined, indicating that all WAVEs function in a similar manner.

Then, to examine the importance of VPH domain in actin clustering, the ΔVPH mutants of full-length WAVEs were expressed in COS-7 cells. The ΔVPH mutants of WAVE2 and WAVE3 did not induce filamentous actin as well as ΔVPH mutant of WAVE1 (Fig. 4). In the consequence, VPH domain was demonstrated to be essential for WAVE family proteins to function on actin cytoskeleton. The localizations of ΔVPH WAVEs were different with one another in COS-7 cells. However, this difference was not observed when WAVEs were expressed in other cultured cells such as NIH 3T3 (not shown). Therefore this different localizations of ΔVPH WAVEs seen in COS-7 cells did not seem to be important for distinguishing their characters.

DISCUSSION

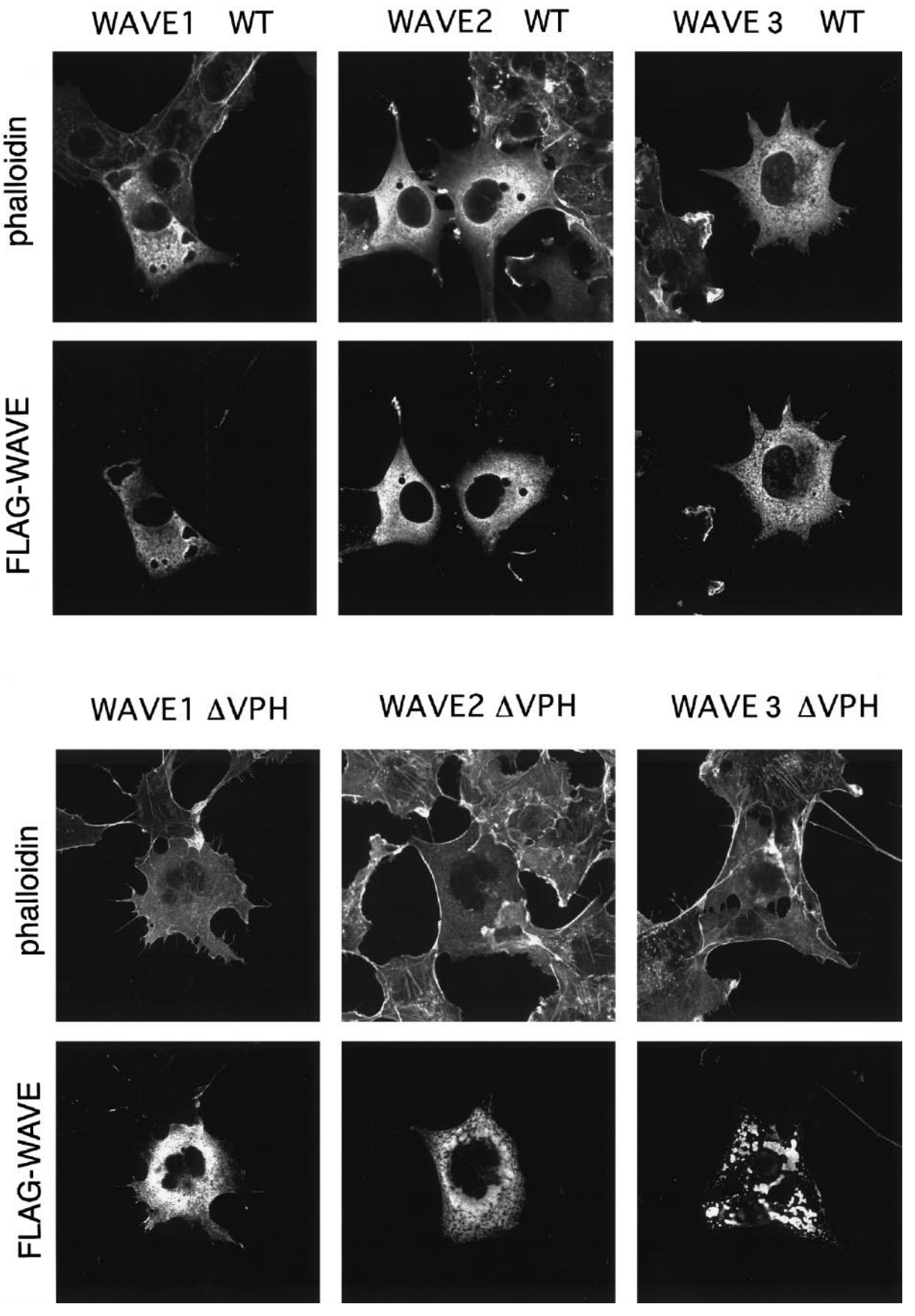
Here we identified two human WAVE/SCAR homologues, which have 48% amino acid sequence identity. These three WAVE proteins seem to cover most of organs. Bear *et al.* (9) mentioned the existence of the fourth WAVE family gene, SCAR4, but SCAR4 was found to be the same to the C-terminal half of WAVE2/SCAR2 gene by this study.

WAVE1, WAVE2, and WAVE3 are composed of almost identical domains. All WAVEs have SHD (SCAR homology domain), basic domain, proline-rich region, VPH (Verproline homology) domain, and acidic domain. Especially at their SHD and C-terminal region containing VPH domain and acidic domain, the amino acids are highly conserved, showing the sequence identity of 75 and 80%, respectively (Fig. 1).

Now, we do not know the function of the SHD. However, since SHD has a putative leucine-zipper-like motif as shown by circles in Fig. 1A, some important regulatory molecules might associate through this region.

VPH domain, a direct actin binding domain, and acidic domain, a region required for Arp2/3 binding, are well conserved in all WASP family proteins including Bee1 (18), WASP, N-WASP, WAVE1, WAVE2, and WAVE3, making these domains a determinant of this family. Many groups demonstrated that both VPH domain and acidic domain are required for reconstituting

FIG. 4. Ectopic expression of full-length WAVEs in COS-7 cells. Both wild type and ΔVPH mutant of FLAG-tagged WAVE1, WAVE2, and WAVE3 were transiently expressed in COS-7 cells. Thirty hours after transfection, the cells were fixed and stained with phalloidin to visualize actin filaments and antibody against FLAG to visualize ectopically expressed WAVEs.


WAVE1 WT
WAVE2 WT
WAVE 3 WT
phalloidin
FLAG-WAVE
WAVE1 ΔVPH
WAVE2 ΔVPH
WAVE 3 ΔVPH
phalloidin
FLAG-WAVE

enhanced actin polymerization *in vitro*. VPH domain alone, or acidic domain alone was not sufficient for this reconstitution (11, 12). In fact, VPH domain from all WAVE proteins was shown to be essential for inducing actin clusters in COS-7 cells, also supporting the importance of this domain.

Collectively, the high conservation of WAVEs at N- and C-termini indicates that these WAVE proteins might be under common regulatory signaling pathway and function for similar cellular events.

In contrast to high conservation seen in N- and C-termini, the regions in the middle of the WAVEs, that contains basic domain and proline-rich region, have weak similarity. The natures of amino acids composing of the middle region are similar, but the sequences and the length of these region are various (Fig. 1A), suggesting that different characteristics of these proteins might result from these regions. Proline-rich sequence has been demonstrated to associate with proteins with SH3 domain (19–21) and profilin (22). Thus, different binding proteins between WAVEs might associate through these regions.

Wiskott-Aldrich syndrome (WAS) is a disease in immune system, probably due to defects in regulation of actin cytoskeleton. Thus, it is interesting that WAVE2 mapped very near to WASP gene from two reasons (Fig. 2B). First, both WASP and WAVE2 were expressed in peripheral blood leukocytes. And second, WAVE has been suggested to play a role in regulation of actin cytoskeleton (8, 9). Indeed, the experiment of WASP deficient mice showed that the defect of WASP gene alone can not explain the all malfunctions of peripheral bloods in Wiskott–Aldrich syndrome (23). Collectively, mutation in WAVE2 gene might be another factor that cause Wiskott-Aldrich syndrome in cooperation with mutated WASP itself.

In this study, we identified two novel WAVE/SCAR homologues. The finding of two WAVE family genes which cover most of organs suggest that WAVEs might have general function for regulating actin cytoskeleton.

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