Cell Motility and the Cytoskeleton (2009)

The Wave Complex is Intrinsically Inactive

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The Wave proteins activate the Arp2/3 complex at the leading edge of migrating cells. The resulting actin polymerization powers the projection of the plasma membrane in lamellipodia and membrane ruffles. The Wave proteins are always found associated with partner proteins. The canonical Wave complex is a stable complex containing five subunits. Even though it is well admitted that this complex plays an essential regulatory role on Wave function, the mechanisms by which Wave proteins are regulated within the complex are still elusive. Even the constitutive activity or inactivity of the complex is controversial. The major difficulty of these assays resides in the long and difficult purification of the Wave complex by a combination of several chromatography steps, which gives an overall low yield and increases the chance of Wave complex denaturation. Here we report a greatly simplified approach to purify the human Wave complex using a stable cell line expressing a tagged subunit and affinity chromatography. This protocol provided us with sufficient amount of pure Wave complex for functional assays. These assays unambiguously established that the Wave complex in its native conformation is intrinsically inactive, indicating that, like WASP proteins, Wave proteins have a masked C-terminal Arp2/3 binding site at resting state. As a consequence, the Wave complex has to be recruited and activated at the plasma membrane to project migration structures. Importantly, the approach we describe here for multiprotein complex purification is likely applicable to a wide range of human multiprotein complexes. Cell Motil. Cytoskeleton 2009.

Key words: Arp2/3 complex; actin; multiprotein complex; stable cell line; cell migration

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Abbreviations used: mAb, monoclonal Antibody; NPF, Nucleation Promoting Factors; ORF, Open Reading Frame; PC, Protein C epitope; WCA, WH2-Connector-Acidic.

Contract grant sponsor: Fondation pour la Recherche Médicale; Contract grant number: INE20071110919; Contract grant sponsor: CNRS (PEPS).

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Received 13 November 2008; Revised 29 December 2008; Accepted 12 January 2009

Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cm.20342

INTRODUCTION

Cell migration involves actin polymerization at the front of the cell. Actin polymerization plays an essential role in powering the projection of the plasma membrane in structures such as lamellipodia and ruffles [Takenawa and Suetsugu, 2007]. Most of this actin polymerization is contributed by the Arp2/3 complex, which nucleates actin filaments off preexisting filaments [Pollard, 2007]. This process induces the formation of an array of branched actin filaments. The Arp2/3 complex is intrinsically inactive and needs to be activated by Nucleation Promoting Factors (NPFs). NPFs are characterized by a C-terminal WCA domain (WH2-Connector-Acidic). The WCA domain binds actin and the Arp2/3 complex and is sufficient to trigger Arp2/3 dependent actin polymerization in vitro.

Wave proteins are the major NPFs in lamellipodia and ruffles. Wave proteins are localized at the tip of these structures [Miki et al., 1998; Hahne et al., 2001; Innocenti et al., 2004; Steffen et al., 2004]. Genetic inactivation of Wave genes in the mouse impairs their formation [Yamazaki et al., 2003; Yan et al., 2003]. Mammalian cells express three genes encoding Wave proteins (also known as Scar proteins). Wave2 is the most ubiquitous protein, whereas Wave1 and Wave3 have more restricted expression patterns [Sossey-Alaoui et al., 2003]. Wave1 and Wave3 are well expressed in the brain. Interestingly, in cells coexpressing Wave1 and 2, a functional specialization was identified with Wave1 being required for the formation of dorsal ruffles and Wave2 for peripheral ruffles [Suetsugu et al., 2003].

Formation of lamellipodia and ruffles is under the control of the small GTPase Rac. Wave proteins are essential downstream factors for this effect [Miki et al., 1998; Innocenti et al., 2004; Steffen et al., 2004]. But how Rac regulates Wave proteins is still unclear. IRSp53, a protein bridging Rac to Wave2 has been first isolated [Miki et al., 2000]. This protein directly binds membranes and might be able to sense or induce a specific curvature of the plasma membrane [Scita et al., 2008]. A second route to connect Rac to Wave involves the so-called Wave complex. The purification of Wave1 from brain has revealed that Wave1 was associated with four other subunits in the Wave complex [Eden et al., 2002; Kim et al., 2006]. The purification of Wave2 from HeLa cells revealed a similar complex [Gautreau et al., 2004; Innocenti et al., 2004]. Wave3 also likely incorporates into such a complex [Stovold et al., 2005]. Importantly, these complexes contained the subunit CyFIP1, also known as Sra1 (Specifically Rac Associated-1), a previously known effector of Rac [Kobayashi et al., 1998]. In addition to Rac signalling, phosphorylation events also regulate Wave activity [Kim et al., 2006; Takenawa and Suetsugu, 2007]. To understand signaling through the Wave proteins, one must understand their off state, their on state and how they transit between these two states.

Wave molecules in isolation activate the Arp2/3 complex in a constitutive manner [Machesky et al., 1999; Eden et al., 2002; Innocenti et al., 2004]. They differ in that respect from WASP molecules, which are autoinhibited through an intramolecular interaction masking their C-terminal Arp2/3 binding site [Takenawa and Suetsugu, 2007]. Regulation of Wave activity relies on its multiprotein complex. This multiprotein complex behaves as a biochemical entity, since depletion of one subunit through RNAi or genetic inactivation leads to degradation of the remaining subunits, in a variety of plant and animal organisms [Blagg et al., 2003; Kunda et al., 2003; Innocenti et al., 2004; Steffen et al., 2004; Le et al., 2006; Derivery et al., 2008]. The Wave1 complex purified from brain was reported to be inactive, and to dissociate upon GTP-bound Rac addition, thus releasing active Wave1 [Eden et al., 2002]. According to these results, partner subunits have a primary inhibitory role on Wave activity. The activation model was however challenged by the lack of dissociation of the Wave2 complex in cell-free extract upon active Rac addition [Innocenti et al., 2004]. Moreover, all subunits of the Wave2 complex are localized at the lamellipodium tip, where Wave proteins are active [Steffen et al., 2004]. These controversial findings could simply indicate that the activation mechanism of Wave2 is different from the one of Wave1, but actually even the intrinsic inactivity of the Wave complex was challenged. The global architecture of the Wave2 complex has been deciphered [Gautreau et al., 2004; Innocenti et al., 2004]. Sequential addition of Wave2 complex subunits modulates but does not suppress Wave2 activity [Innocenti et al., 2004]. Furthermore, purified Wave1 complex from brain was recently found to be active, contrary to the original observation [Kim et al., 2006]. These results suggested that the Wave complex was intrinsically active and was recruited from the cytosol to the plasma membrane in an already active form. This idea was tested by fractionating cells and by assaying immunoprecipitated Wave2 from cytosolic and membrane fractions. The cytosolic pool of the Wave complex was inactive whereas the membrane pool was active [Suetsugu et al., 2006]. This observation contradicts the proposed constitutive activity of the Wave complex.

To recapitulate, the Wave complex was inactive in two publications [Eden et al., 2002; Suetsugu et al., 2006] and active in two others [Innocenti et al., 2004; Kim et al., 2006]. To understand the molecular mechanisms of Wave activation during cell migration, one needs first to solve this controversy on the intrinsic activity of the Wave complex. We thus chose this objective,

while trying to understand where discrepancies might come from.

Combination of different paralogous subunits creates a diverse repertoire of Wave complexes. The canonical Wave complex contains:

- the CyFIP1 subunit, or its paralogous protein CyFIP2 (also known as PIR121);
- the Nap1 subunit, or its paralogous protein Hem1 in hematopoietic cells;
- one of the three Wave subunit;
- the Abi1 subunit, or one of its paralogous protein, Abi2 or NESH; and
- the Brk1 subunit (also known as Hspc300), which is the only one that does not have a paralogous protein in any species examined.

Just the combination of the paralogous subunits can create as many as 36 different pentameric complexes, but the Abi genes provide an additional source of complexity due to their extensive alternative splicing [Ziemnicka-Kotula et al., 1998]. Wave complexes purified from a tissue or even a single cell line are thus a mixture of Wave complexes assembled from the expressed paralogous subunits [Eden et al., 2002; Gautreau et al., 2004; Innocenti et al., 2004; Kim et al., 2006]. If the diversity of mammalian Wave complexes certainly increase the possible ways to regulate the three Wave activities, it is also a likely cause of discrepancies.

The other potential pitfall in studying multiprotein complexes comes from their purification. To purify endogenous multiprotein complexes from cells or tissues, many steps of chromatography are typically required and this gives an overall low yield. For the Wave1 and 2 complexes, between four and eight steps are required [Eden et al., 2002; Gautreau et al., 2004; Kim et al., 2006]. The combination of several steps in different physico–chemical conditions, in a procedure that lasts several days, increases the likelihood of denaturation. An alternative to the purification of an endogenous complex is the reconstitution of this complex from recombinant proteins. This approach is also long and difficult. Moreover, a reconstitution is successful when it provides a recombinant multiprotein complex that behaves like the native complex. The native behavior in the case of the Wave complex is precisely unclear and represents our goal. So we decided to choose an intermediate approach, where a recombinant tagged subunit is expressed in a stable cell line and the endogenous complex assembled around this recombinant subunit is purified efficiently and rapidly by affinity chromatography. Using this strategy, we report here a greatly improved protocol for Wave complex purification. We found that the purified Wave complex is inactive.

MATERIALS AND METHODS

Plasmids

All mammalian expression plasmids were derived from pCDNA5/FRT/V5-His (Invitrogen). All the Open Reading Frames (ORFs) were derived from human cDNAs and flanked by FseI and AscI sites for easy shuttling between compatible plasmids. CyFIP1 (GenBank accession no. XM 039225), Nap1 (GenBank accession no. AB011159), Wave2 (GenBank accession no. AB026542), and Brk1 (GenBank accession no. BC019303) were previously described [Gautreau et al., 2004]. Abi1_1 (GenBank accession no. BC024254) was amplified by PCR from cDNA clone IMAGE:3531592, Abi1_2 (GenBank accession no. FJ380057) was amplified from cDNA clone IMAGE:5288756, Abi1_3 (GenBank accession no. FJ380058) was amplified from cDNA clone IMAGE:4825581, Abi1_4 (GenBank accession no. FJ380059) was amplified from an HeLa library of cDNA. These plasmids tag the ORF in its N-terminus by either FLAG-HA (MDYKDDDDK-YPYDVPDYA) or by PC (MEDQVDPRLIDGKEFDGRP) followed by a TEV binding and cleavage site (DYDIPTTENLYFQG). The V5-His tags in the C-terminus were not translated because of the natural stop codon of the ORFs kept before the AscI site. All constructs were verified by sequencing both strands of DNA. Full length human Wave2 was also cloned in a pET28 vector, tagging the protein by a N-terminal $(His)_6$ peptide, for bacterial expression. pGEX Rac1 Q61L and Nck SH3.1 were kindly provided by Alan Hall (Memorial Sloan-Kettering Cancer Center) and Bruce Mayer (University of Connecticut Health Center), respectively.

Cell Culture

Flp-In™ T-REx™ 293 cells (Invitrogen) were grown in DMEM supplemented with 10% FCS. The stable expression of Tet Repressor was not used in this study and thus was not selected for using blasticidin. They were transfected using standard calcium phosphate method. Briefly, 250,000 cells (plated the day before) were cotransfected with 1 μg of pOG44 plasmid (Invitrogen) and 0.2 μg of plasmid of interest. Stable transfectants obtained by homologous recombination at the Flp-In site were selected using 200 $\mu g \cdot mL^{-1}$ Hygrogold (Invitrogen). Large scale cultures were grown using 2 L spinner flasks (Techne) in DMEM supplemented with 10% FCS, 100 $\mu g \cdot mL^{-1}$ Hygrogold and 1% Penicillin-Streptomycin. Briefly, eight 15-cm dishes of cells were grown up to 90% confluency, trypsinized and diluted into 1 L of medium in one spinner. Three days after, culture was splitted into four spinners and volume was adjusted to 1 L. Two days after, volume was adjusted to 1.5 L and after two more days, cells were harvested,

washed in XB buffer (20 mM Hepes, 100 mM KCl, pH 7.7). Cell pellets were then thrown into liquid nitrogen and stored frozen at −80°C.

SDS-PAGE and Western Blot

SDS-PAGE was performed using NuPAGE 4–12% Bis-Tris gels (Invitrogen) and MES running buffer. Gels were transferred on nitrocellulose membranes according to the manufacturer's instructions. Western blots were revealed using HRP coupled antibodies, Supersignal kit (Pierce) and a Fuji LAS-3000 (Fujifilm). Colloidal Coomassie blue (Invitrogen) was used for total protein staining of the gel. Protein concentrations were determined after SDS-PAGE using densitometry of the bands and purified actin as a standard. Quantifications were performed with Image J software.

Antibodies

A rabbit polyclonal antibody (W2C) targeting the last 15 amino-acids of human Wave2 (SEDDSSEFDEDDWSD), the Acidic domain, was generated (Zymed) and purified by affinity with the immunogenic peptide. Affinity purified polyclonal antibodies targeting peptides derived from Wave2 (W2), Sra1, and specifically an alternative exon of a long Abi1 isoform (peptide SSGGYRRTPSV) were previously described [Gautreau et al., 2004]. A serum obtained after immunization with peptide HGNNQPARTGTLSRTNP present in many Abi1 isoforms was kindly provided by Ann Marie Pendergast [Courtney et al., 2000]. Abi2 (P20) polyclonal antibody was from Santa Cruz. FLAG M2 mAb was from Sigma.

Purification of Recombinant Proteins

Unless stated otherwise, all purification steps were performed at 4°C. $(His)_6$-tagged full length Wave2 expression in *E coli* BL21 (DE3) Star strain (Invitrogen) was induced for 2 h at 20°C with 1 mM IPTG. Cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) supplemented with Protease Inhibitor Cocktail (Sigma). After sonication and centrifugation (10,000 × *g*, 30 min), the supernatant was incubated with Ni-NTA resin (Qiagen), washed in (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) then eluted in (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Fractions containing the protein were pooled, dyalized against PBS, flash frozen in liquid nitrogen and kept at −80°C until use. GST-Rac1 L61 (active mutant) was produced and purified in *E. coli* as fusions with GST, and then Rac1 was cleaved off GST using thrombin as previously described [Haeusler et al., 2006]. GST and GST fused to the first SH3 domain of Nck (GST-Nck SH3.1) were purified in *E. coli* using a standard protocol.

Purification of Tagged Wave Complexes

For small scale purification of FLAG-HA tagged Wave complexes, two 15 cm-dishes of 293 cells (6×10^7 cells) were lysed in 1 mL of RIPA buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% DOC, 0.1% SDS, pH 7.7) supplemented with Protease Inhibitor Cocktail (1:1000, Sigma) and AEBSF 1 mM (Interchim). The extract was clarified by centrifugation (20,000 × *g*, 10 min) then incubated with 20 μL of FLAG M2 agarose beads (Sigma) for 4 h with rocking. After two washes in RIPA and 2 washes in XB (20 mM Hepes, 100 mM KCl, pH 7.7), proteins were eluted in 20 μL of XB supplemented with 0.1 mg/mL of 3× FLAG peptide (Sigma) overnight, and then analyzed by SDS PAGE.

For large scale purification of FLAG-tagged Wave complexes, 6 L of 293 cells expressing FLAG-Abi1_4 (10^{10} cells, about 20 mL of cell pellet) were lysed in 30 mL of RIPA buffer supplemented with Protease Inhibitor Cocktail (1:500) and AEBSF 2 mM and rocked for 1 h at 4°C. The extract was clarified by centrifugation (3300*g*, 10 min) and ultracentrifugation (200,000 × *g*, 1 h). The extract was filtered through a 0.45 μm filter (Millipore) then applied on a column containing 1 mL of FLAG-M2 agarose beads with recirculation for 4 h at 4°C. Beads were washed extensively with 50 mL of RIPA, 50 mL of XB, then 50 mL of (50 mM PIPES, 150 mM KCl pH 6.8). Several elutions were then performed by mixing the resin and 1 mL of the appropriate buffer. The first elution was done in (50 mM PIPES, 150 mM KCl, 1 mM DTT, Protease Inhibitor Cocktail (1:1000), 0.1 mg/mL 3× FLAG peptide, pH 6.8), the second in (50 mM PIPES, 50 mM KCl, 1 mM DTT, pH 6.8) and the last two in (50 mM PIPES, 1 mM DTT, pH 6.8). Each elution was performed by rocking the slurry (30 min at 17°C then overnight at 4°C for the first one, 10 min at 4°C for the others) and centrifuging at 300 × *g* for 1 min. Elution fractions were then pooled, giving a final KCl concentration of 50 mM. This eluate was then clarified (100,000 × *g*, 30 min) and applied on a miniS PC 3.2/3 column driven by a Smart System (Pharmacia). Elution was performed using a linear gradient between (50 mM PIPES, 50 mM KCl, 1 mM DTT, pH 6.8) and (50 mM PIPES, 2 M KCl, 1 mM DTT, pH 6.8). For analytical gel filtration analysis, 20 μL of the miniS peak (roughly 20 μg of Wave complex) was loaded on a Superdex 200 PC 3.2/3 column driven by a Smart System (Pharmacia) at a flow rate of 40 μL s^{-1} in PBS. The stability assay for the Wave complex was performed with 20 μL of FLAG eluate in different buffers. Each immunoprecipitation was performed using about 6×10^7 cells expressing FLAG-Abi1_4. The eluates were then spun in the TLA100 rotor at 40,000 rpm for 30 min. Apparent K factor for centrifuging 20 μL at 40,000 rpm in this rotor is 24.25. This K

factor implies that 2.2 h are required to pellet the native Wave complex of 11 S. Thus the pellet after the 30 min spin contains only aggregated Wave complex. Supernatant and pellets were analyzed by SDS-PAGE and Coomassie staining.

For large scale purification of PC- tagged Wave complex, 3 L of cells expressing PC-TEV-Abi1_4 (5×10^9 cells, 10 mL of cell pellet) were lysed in 40mL of (50 mM Hepes, 200 mM NaCl, 1mM CaCl2, 1% NP-40, 0,5% DOC, 0.1% SDS, 5% glycerol, pH 7.7) supplemented with Protease Inhibitor Cocktail (1:500) and AEBSF 2 mM and rocked for 1 h at 4°C. The extract was clarified by centrifugation (3300*g*, 10 min) and ultracentrifugation (200,000 × *g*, 1 h). After filtration through a 0.45 μm filter (Millipore), the extract was applied on a column containing 1 mL of Protein C affinity beads (Roche) with recirculation for 4 h. Beads were washed extensively with 50 mL of lysis buffer, 50 mL of (50 mM Hepes, 200 mM NaCl, 1 mM $CaCl_2$, 20% glycerol, pH 7.7), 50 mL of (50 mM Hepes, 400 mM NaCl, 1 mM $CaCl_2$, 20% glycerol, pH 7.7), 50 mL of (50 mM Hepes, 200 mM NaCl, 20% glycerol, pH 7.7). Several elutions were then performed by mixing the resin with 1mL of (50 mM Hepes, 200 mM NaCl, 5 mM EGTA, 20% glycerol, pH 7.7). Each elution was performed by rocking the slurry (overnight at 4°C for the first one, 10 min at 4°C for the other ones) then centrifuging at 300 × *g* for 1 min. Elution fractions were pooled and concentrated on Amicon ultra (MWCO 3 kDa, Millipore).

Molecular Mass of the Wave Complex

Molecular mass was determined from the experimentally measured Stokes' radius and sedimentation value using the Siegel and Monty equation [Siegel and Monty, 1966]:

$$M = 6\pi\eta\ NaS/(1 - v\rho),$$

where M is the molecular mass in Da ($g \cdot mol^{-1}$); η is the viscosity of the medium, approximated by water, $1.002 \times 10^{-2}\ g \cdot cm^{-1} \cdot sec^{-1}$; N is Avogadro's number, $6.022 \times 10^{23}\ mol^{-1}$; a is the Stokes' radius (Å, i.e. 10^{-8} cm); S is the sedimentation value (S en Svedberg, 10^{-13} s); *v* is the partial specific volume, $0.725\ cm^3 \cdot g^{-1}$; ρ is the density of medium, $0.998\ g \cdot cm^{-3}$.

Mass Spectrometry

Gel slices were reduced, alkylated and subjected to digestion with trypsin (Roche Diagnostics). Extracted peptides were dried and resolubilized in solvent A (95/5 water/acetonitrile in 0.1% (w/v) formic acid). The total digestion product of a gel slice was used per LC-MS/MS analysis. The extracted peptides were concentrated and separated on a LC-Packings system (Dionex S.A.) coupled to the nano-electrospray II ionisation interface of a QSTAR Pulsar i (Applied Biosystems) using a PicoTip (10 μm i.d., New Objectives). HPLC mobile phases contained for solvent A and for solvent B (20/80: water/acetonitrile in 0.085% (w/v) formic acid). Bound peptides were eluted with a gradient of 5–50% of solvent B in 60 min. Information-dependent acquisition was used to acquire MS/MS data, with experiments designed such as the two most abundant peptides were subject to collision-induced dissociation, using nitrogen as collision gas, every 5 s. Data were searched using MASCOT (Matrix Science) software on the NCBI nr *Human* database. All data were manually verified in order to minimize errors in protein identification and characterization.

Pyrene-Actin Polymerization Assay

Actin polymerization was followed by changes in pyrene fluorescence using a Xenius SAFAS fluorimeter (Safas SA, Monaco). Actin was isolated from rabbit skeletal muscle acetone powder as described [Spudich and Watt, 1971] and stored in G buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.1 mM CaCl2, and 0.1 mM DTT 0.01% NaN_3). Arp2/3 complex was purified as described [Egile et al., 1999] or purchased from Cytoskeleton. Briefly, MgATP-G-actin was prepared by incubating CaATP-G-actin (10 μM, 10% pyrene labeled) in G buffer with 200 μM EGTA and 20 μM $MgCl_2$ for 5 min on ice. The resulting MgATP-G-actin was used within 1 h. Polymerization of 2.5 μM MgATP-G-actin was initiated by the addition of one-tenth volume of 10× KMEI (500 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, and 100 mM imidazole-HCl, pH 7.0). For the control curves, an equal amount of buffer without protein was always included. Handling of data and design of graphs was performed using Kaleidagraph v3.6 software (Synergy Software, Reading, PA).

Immunoprecipitations

One microgram of native or denatured PC tagged Wave complex diluted in 1 mL of buffer A (Hepes 50 mM, KCl 100 mM, pH 7.7) was immunoprecipitated with 3 μg of non immune Rabbit IgG, W2 or W2C antibodies and 10 μL of Protein G beads (Pierce) during 2 h of rocking at 4°C. Beads were washed four times with 1 mL of buffer A and analyzed by SDS-PAGE.

RESULTS

Characterization of Stable Cell Lines Expressing Tagged Subunits of the Wave Complex

To purify efficiently the Wave complex assembled around a tagged subunit, we first decided to compare the different tagged subunits for their ability to incorporate

into the endogenous Wave complex. We focused on the so-called ubiquitous Wave complex, the major Wave complex expressed by Hela cells [Gautreau et al., 2004]. So we cloned CyFIP1, Nap1, Wave2, Abi1, and Brk1 into a plasmid encoding a fusion with the two epitope tags FLAG and HA. In this study, the presence of the HA epitope was not used, but it potentially allows Tandem Affinity Purification, as previously described [Groisman et al., 2003]. Because Abi1 is spliced alternatively, we decided to compare four different splice variants obtained as cDNAs of genes expressed in different tissues or HeLa cells. With this series of constructs, we obtained a collection of stable cell lines derived from 293 cells using homologous recombination. As a result, all tagged subunits are expressed from a single well expressed locus. To check whether these tagged subunits were able to integrate into the endogenous Wave complex, we performed immunoprecipitations from the collection of lines using FLAG mAb followed by elution using 3× FLAG peptide competition. The eluates were analyzed by SDS-PAGE and Coomassie staining (Fig. 1A). All eluates displayed the characteristic pattern of the Wave complex with the high molecular weight doublet CyFIP and Nap1. It should be noted that the different cell lines grew at somewhat different rates and despite an attempt to equalize the number of cells used for this experiment, the immunoprecipitation from the different lines was not performed with an equivalent number of cells. As a consequence, this experiment was not the basis to select the best cell line for Wave complex purification. Nonetheless, this experiment demonstrates the feasibility of the purification of Wave complexes tagged on any subunit.

To select for the line producing the largest amount of tagged Wave complexes as a starting material for purification, we first compared their level of Wave2. None of them displayed a significantly increased level, suggesting that no single subunit is the limiting factor controlling the level of Wave complexes. We thus decided to estimate the proportion of Wave complexes associated with the tagged subunit or the endogenous one. The tagged subunit was depleted by a single round of immunoprecipitation and the depleted lysates were compared to the initial lysates by the depletion of endogenous Wave2. The highest depletion of Wave2 was obtained upon FLAG depletion of Abi1_4, the shortest splice variant of Abi1 (Fig. 1B). Interestingly, the expression of FLAG-Abi1_4 replaced the endogenous Abi proteins, since both the major endogenous Abi1, a longer isoform than Abi1_4, and Abi2 were profoundly down-regulated. This effect was obvious when initial lysates of cells expressing Abi1 isoforms were compared to the ones of cells expressing CyFIP1 or Nap1. This 'molecular replacement' of endogenous subunits by the recombinant

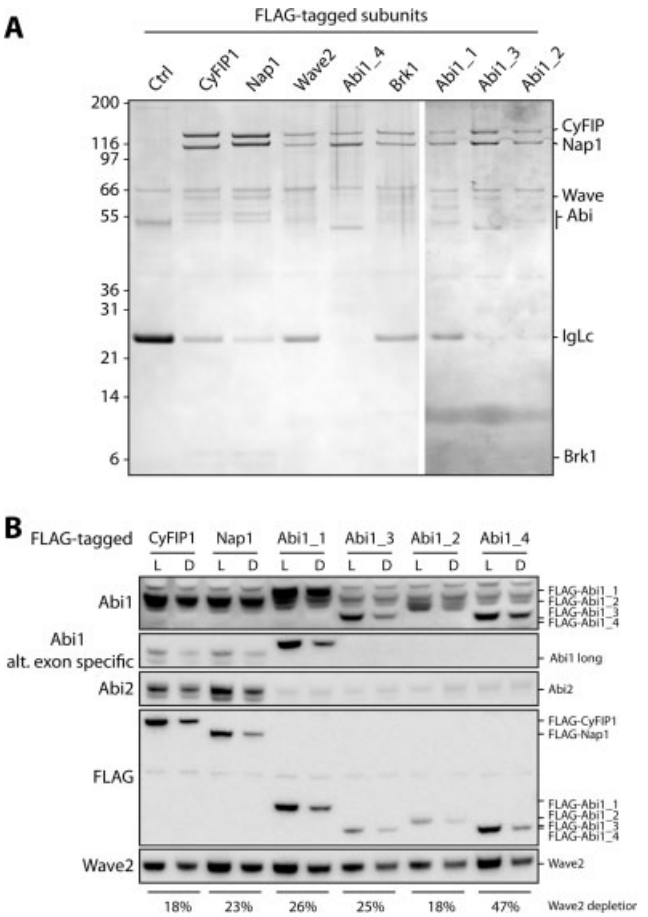

Fig. 1. Characterization of stable 293 cell lines expressing FLAG-tagged subunits of the Wave complex. **(A)** Small-scale affinity purification of Wave complexes tagged on the different subunits. This Coomassie stained gel of eluates from the FLAG affinity resin indicates that all tagged subunits integrate into the Wave complex. The presence of the Wave complex is best revealed by the high molecular weight subunits CyFIP and Nap1. The positions of Wave, Abi and Brk1 are indicated, even though they are difficult to see in small scale purifications. Abi1_1–_4 refers to four different splice variants of Abi1. Note that the addition of the tag decreases slightly the electrophoretic mobility of subunits. A variable number of FLAG beads were carried over in these small scale purifications as indicated by the light chain of the FLAG antibody (IgLC). Positions of molecular markers (in kDa) are indicated on the left of the gel. **(B)** Analysis of subunit expression and depletion upon FLAG immunoprecipitation in different cell lines. Lysates before (L) or after FLAG depletion (D) were analyzed by western blot with the indicated antibodies. Abi1 was analyzed both with an antibody recognizing all forms of Abi1 and an antibody recognizing specifically an alternative exon of Abi1 present in long isoforms such as Abi1_1. The expression of all tagged subunits was detected with FLAG antibodies. A significant depletion of endogenous Wave2 was observed with all six constructs indicating that the tag was exposed in all cases. This depletion was estimated by densitometry and given in % below the western blot. FLAG-Abi1_4 shows the best incorporation. Furthermore, expression of a particular Abi1 replaces endogenous Abi1 and Abi2 subunits.

one probably reflects that unincorporated subunits are degraded and that endogenous subunits are overwhelmed by the recombinant one.

The cell line expressing tagged Brk1 was not selected, because Brk1 forms a free pool in addition to the Wave complex [Gautreau et al., 2004]. The cell line expressing Wave2 grew significantly slower than the others. For this reason, this line was not selected as a good starting material for Wave complex purification. The cell line expressing Abi1_4 appeared as the best starting material, because it grows well, tagged Abi1_4 is very well integrated into the endogenous Wave complex, and this cell line has a simplified repertoire of expressed Abi subunits.

Optimization of Wave Complex Purification

We next established the purification protocol of the Wave complex from the 293 cell line expressing FLAG tagged Abi1_4. We grew several liters of this cell line in suspension using spinner bottles. A cell lysate was prepared and incubated with beads conjugated with the FLAG mAb. In this large scale immunoprecipitation, depletion of tagged Wave complexes was essentially complete but elution with the competing peptide proved to demand optimization. We found that temperature was a critical determinant of elution efficiency. Increasing the temperature promotes elution, but the temperature has to be maintained within a reasonnable range to avoid denaturation of the complex. We found that a 30 min incubation at 17°C following an overnight incubation at 4°C was a good trade-off, providing 75% elution. The eluted material was obviously enriched in Wave complex, since its characteristic pattern could be clearly distinguished (Fig 2A, lane input), despite the presence of several contaminants in minor amounts. The repertoire of Wave complex subunits purified with FLAG-Abi1_4 was analyzed by LC-MS/MS (Table I). Both CyFIP1 and 2 were present. These two proteins are 87% identical explaining why seven peptides were common. In addition, 10 peptides were specific to CyFIP2, and one was specific to CyFIP1, suggesting that CyFIP2 was more abundant than CyFIP1. The presence of Nap1 was confirmed by 12 peptide and there was no peptide from its hematopoïetic specific paralogue Hem1. A mixture of Wave1 and 2 was present. In the upper band of the doublet, Wave1 was represented by six peptides and Wave2 by only one. In the lower band of the doublet, the ratio was inverted with three peptides of Wave2 and a single one of Wave1. Despite this enrichment of Wave1 in the upper band and of Wave2 in the lower band, the relative intensity of these two bands cannot be used as an indicator of the proportion between these two Wave paralogues, given that these proteins are subjected to band shifts upon phosphorylation. As expected, all the Abi peptides belonged to the tagged splice variant of Abi1 we expressed and purified.

To further purify the Wave complex, we used an ion exchange column. The main contaminant in this preparation is the acidic 3× FLAG peptide. The FLAG eluate was thus loaded on a miniS cation exchange column. The Wave complex was specifically retained but the 3× FLAG peptide went through. After elution with a linear gradient of salt, the Wave complex was highly purified and concentrated (Fig. 2A). In two steps, FLAG affinity and miniS, we obtained 180 μg of pure Wave complex from 10^{10} cells (6 L culture). This amount of Wave complex is unprecedented in the litterature. For an analytical purpose, we performed size exclusion chromatography with this purified Wave complex. An aliquot fraction was loaded on a calibrated superdex-200 column. The five subunits eluted as expected as a single peak (Fig. 2B). This peak eluted at a position corresponding to a Stokes' radius of 77 Å. This Stokes' radius together with the previously determined sedimentation coefficient (11 S, [Gautreau et al., 2004]) predicts a molecular mass of 348 kDa (see material and methods). This mass was in good agreement with the calculated mass of a complex containing a single molecule of each subunit, which ranges between 380 and 390 kDa depending on the exact composition of paralogs. The smallest subunit Brk1, 9 kDa, was actually shown previously to be a single molecule when bound to Wave2, even though its free form is a trimer [Derivery et al., 2008]. To our knowledge, this is the first experimental determination of the stoichiometry of Wave complex subunits.

Despite the correct yield obtained with the FLAG affinity followed by MiniS cation exchange column, we noticed variable losses of purified Wave complex, suggesting that the conditions used during this purification procedure were not optimal for the stability of the Wave complex. This prompted us to examine the conditions of stability of the Wave complex. We assessed it by eluting the Wave complex with 3× FLAG peptide in different buffers and by ultracentrifuging the eluate in conditions where the native 11 S complex does not pellet. We found that the addition of 20% glycerol improved the solubility of the Wave complex and that low salt conditions were particularly detrimental for Wave complex solubility (data not shown). This last requisite precluded the use of the cation exchange column, which was nonetheless required to get rid of the competing 3× FLAG peptide. We thus decided to change the purification tag. Instead of the FLAG epitope, we used a Protein C (PC) epitope. PC tagged proteins can be purified using a mAb that requires Ca^{2+} for binding and can thus be gently eluted by Ca^{2+} chelation using EGTA.

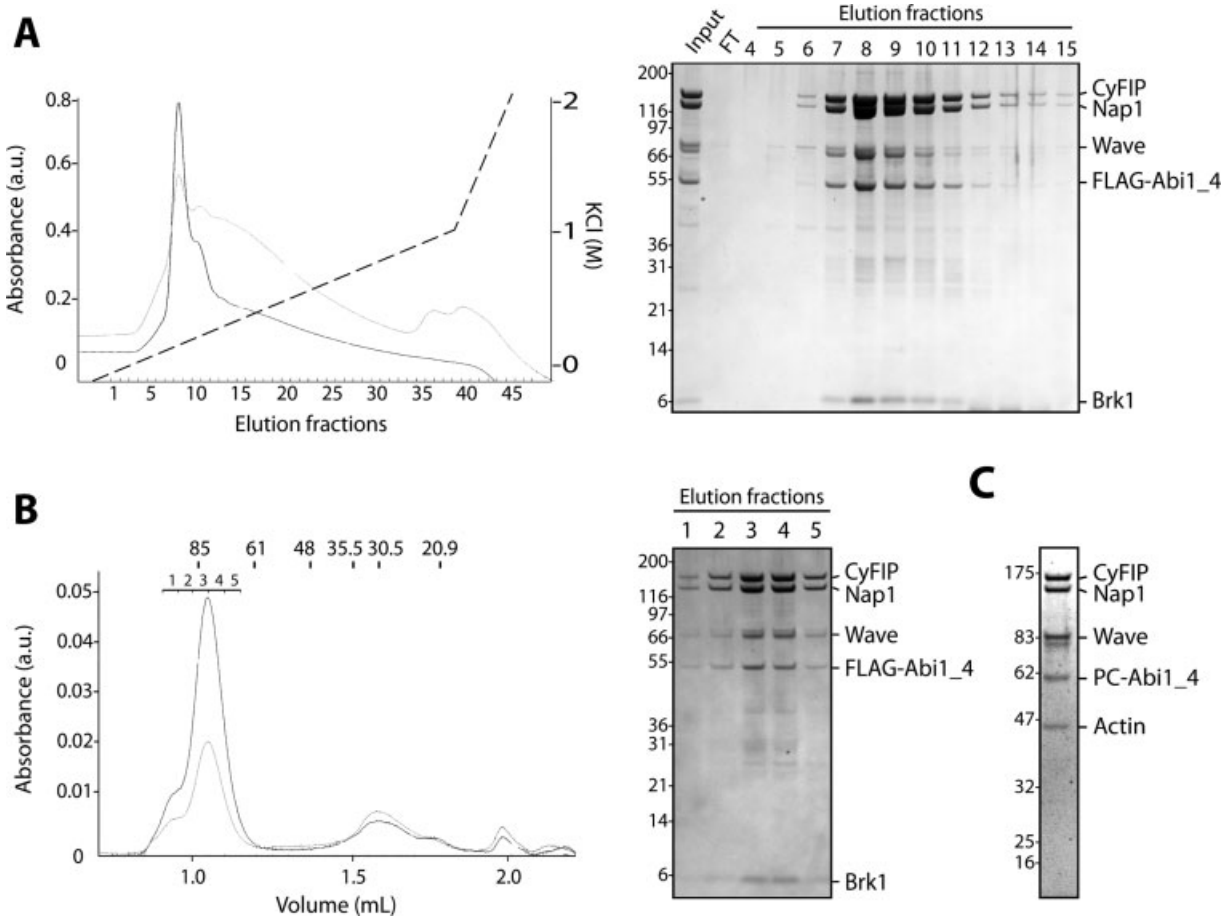

Fig. 2. Purification of the Wave complex containing tagged Abi1_4. (**A**) FLAG affinity purification of the Wave complex was performed at a 100-fold larger scale than in figure 1. The eluate obtained after 3× FLAG peptide competition (Input) was run over a miniS cation exchange column and eluted with a gradient of KCl (dashed line). This ion exchange step is essential to get rid of the 3× FLAG peptide that flows through but is not stained by Coomassie. Profiles of absorbance at 280 nm (dark line) and at 260 nm (gray line) reveal a major protein peak. A Coomassie stained gel (right panel), where input, flow-through (FT) and peak fractions were analyzed, indicated that the Wave complex is responsible for this peak eluting at 200 mM KCl. (**B**) Analytical Size exclusion chromatography on a superdex 200 column further confirms that the five proteins co-eluted from the the MiniS column form indeed a single complex. Positions of standards is indicated by their Stokes' radii in Å above the graph. The Stoke's radius of the Wave complex was determined to be 77 Å. The five fractions of the peak were analyzed by Coomassie blue after SDS-PAGE in the right panel. (**C**) PC affinity purification of the Wave complex. 2 μg of purified complex was analyzed by SDS-PAGE and Coomassie staining. With PC affinity chromatography, actin was additionally retrieved. Molecular weight markers in kDa are indicated on the left of each gel.

We derived a new 293 cell line expressing PC tagged Abi1_4. Using PC affinity chromatography, we could deplete the lysate in tagged Wave complexes and elute 90% of the bound material, while staying throughout the procedure at 4°C in the optimal physico-chemical conditions for Wave complex stability. In a single step, the Wave complex was about 90% pure with a single additional band (Fig. 2C). This band was identified as actin (Table I). Actin is a physiologically relevant partner of the Wave complex. Using the PC affinity protocol but not with the FLAG affinity protocol, actin was retrieved in a stoichiometric proportion with the Wave complex. The yield of Wave complex purification using the single PC affinity step increased more than three times compared with the two step FLAG purification, since we obtained 350 μg from only 5×10^9 cells (3 L culture). This purified Wave complex was used for functional assays.

The Wave complex is Intrinsically Inactive

To assay the activity of the Wave complex, we used the classical pyrene–actin assay in the presence of purified Arp2/3 complex. The Arp2/3 complex is intrinsically inactive and needs to be activated by a NPF. We first compared the activity of the purified Wave complex to the one of recombinant Wave2 in isolation. Recombinant Wave2 potently activated actin polymerization, in agreement with previous experiments using

TABLE I. Identification of Proteins by LC-MS/MS

				Peptides		
Figure	Band	Proteins	No.	Start	End	Sequence
2A Input	CyFIP	CyFIP2	*1*	*50*	*58*	*NAFVTGIAR*
			2	*111*	*121*	*TVEVLEPEVTK*
			3	366	381	YSNSEVVTGSGLDSQK
			4	388	395	ELFDLALR
			5	467	476	MESVFNQAIR
			6	504	514	NVLISVLQAIR
			7	540	548	GGFDIKVPR
			8	*550*	*562*	*AVGPSSTQLYMVR + Oxidation (M:11)*
			9	*563*	*572*	*TMLESLIADK*
			10	*705*	*714*	*LADQIFAYYK*
			11	866	876	TAIPFTQEPQR
			12	950	957	TLIEVMPK
			13	981	989	DIIEYAELK
			14	990	997	TDVFQSLR
			15	*1053*	*1064*	*YAPLHLVPLIER*
			16	*1065*	*1075*	*LGTPQQIAIAR*
			17	1101	1109	SYLQDPIWR
		CyFIP1	*1*	*50*	*58*	*NAFVTGIAR*
			2	*111*	*121*	*TVEVLEPEVTK*
			3	*551*	*563*	*AVGPSSTQLYMVR + Oxidation (M:11)*
			4	*564*	*573*	*TMLESLIADK*
			5	*706*	*715*	*LADQIFAYYK*
			6	867	877	TVLPFSQEFQR
			7	*1054*	*1065*	*YAPLHLVPLIER*
			8	*1066*	*1076*	*LGTPQQIAIAR*
2A Input	Nap1	Nap1	1	312	321	AAEDLFVNIR
			2	358	373	ELATVLSDQPGLLGPK
			3	386	393	DEIIWLLR
			4	480	490	QVEDGEVFDFR
			5	558	570	MFQQCLELPSQSR + Oxidation (M:1)
			6	601	614	SLSLCNMFLDEMAK
			7	874	887	KLVVENVDVLTQMR
			8	875	887	LVVENVDVLTQMR + Oxidation (M:12)
			9	888	902	TSFDKPDQMAALFKR
			10	903	911	LSSVDSVLK
			11	1041	1054	AINQIAAALFTIHK
			12	1063	1074	EFLALASSSLLK
2A Input	Wave upper band	Wave1	1	103	114	SSTIQDQQLFDR
			2	103	115	SSTIQDQQLFDRK
			3	207	223	LAQGPELAEDDANLLHK
			4	499	507	SVLLEAIRK
			5	499	506	SVLLEAIR
			6	527	537	IENDVATILSR
		Wave2	1	230	241	SSTIQDQKLFDR
2A Input	Wave lower band	Wave2	1	79	97	VTQLDPKEEEVSLQGINTR
			2	86	97	EEEVSLQGINTR
			3	463	475	DVVGNDVATILSR
		Wave1	1	499	506	SVLLEAIR
2A Input	FLAG- Abi1_4	Abi1	1	17	28	RALIESYQNLTR
			2	18	28	ALIESYQNLTR
			3	29	44	VADYCENNYIQATDKR
			4	136	154	YIRKPIDYTVLDDVGHGVK
			5	139	154	KPIDYTVLDDVGHGVK
			6	200	219	TLEPVKPPTVPNDYMTSPAR
			7	451	459	VVAIYDYTK
2C	Actin	Beta-actin	1	19	28	AGFAGDDAPR
			2	51	61	DSYVGDEAQSK
			3	96	113	VAPEEHPVLLTEAPLNPK
			4	184	191	DLTDYLMK
			5	197	206	GYSFTTTAER
			6	239	254	SYELPDGQVITIGNER
			7	316	326	EITALAPSTMK
			8	329	335	IIAPPER
			9	360	372	QEYDESGPSIVHR

The common peptides between CyFIP1 and 2 are in italic.

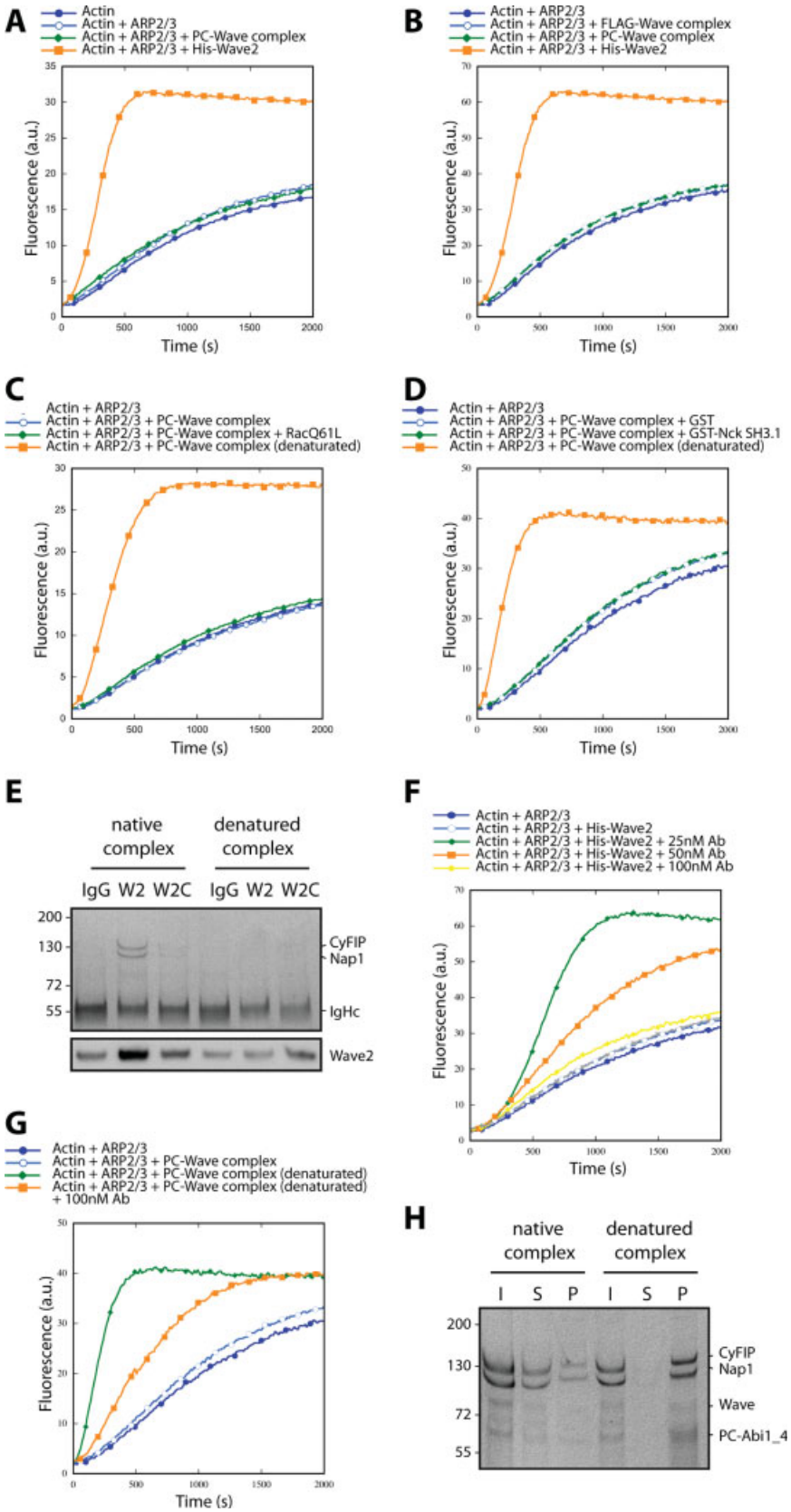

Fig. 3. The Wave complex is intrinsically inactive. (**A**) Wave or Wave complex activity on purified Arp2/3 complex was monitored by pyrene fluorescence of Mg-ATP actin. Conditions: 10 mM imidazole-HCl, pH 7.0, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, and 3 mM NaN_3 at room temperature. 100 nM Wave complex or 100 nM $(His)_6$-Wave2 was added to 43 nM Arp2/3 complex and 2.5 μM actin (10% pyrene labeled). (**B**) The inactivity of the Wave complex does not depend on the purification tag or on freezing of cell pellets. FLAG- and PC-tagged were purified from fresh cells and assayed as in A. (**C**) Rac is not sufficient to activate the Wave complex. Conditions: 200 nM RacQ61L, 43 nM Arp2/3 complex and as in A. Heat denaturation of the Wave complex revealed an activity. (**D**) The first SH3 domain of Nck is not sufficient to activate the Wave complex. Conditions: 200 nM GST or 200 nM GST-Nck SH3.1, 20 nM Arp2/3 complex and as in A. (**E**) The acidic domain of Wave2 is masked within the native Wave complex. Purified Wave complexes were immunoprecipitated using either the W2 antibody targeting a peptide from the central region, or the W2C antibody targeting the last 15 amino-acids of Wave2, the acidic domain. The immunoprecipitates were analyzed with Coomassie staining (upper panel) and Wave2 western blot using W2 antibody (lower panel). Upon denaturation, none of the antibodies is able to immunoprecipitate Wave2 and other subunits of the Wave complex. (**F**) The W2C antibody blocks the activity of purified recombinant Wave2. Conditions: 20 nM Arp2/3 complex and as in A. (**G**) The W2C antibody blocks the activity of denatured Wave complex. Conditions as in A. (**H**) Wave complex subunits aggregate after denaturation. 3 μg of purified Wave complex was subjected to heat treatment or not, centrifuged at low speed, and then total (T), soluble (S) and pellet (P) fractions were analyzed by SDS-PAGE and Coomassie staining.

Wave1 or Wave2 [Machesky et al., 1999; Eden et al., 2002; Innocenti et al., 2004]. In sharp contrast, our purified Wave complex, which contains Wave1 or Wave2, was completely inactive (Fig. 3A). This important conclusion was confirmed using PC tagged Wave complex purified from cells, which were not frozen contrary to our standard protocol (Fig. 3B). Moreover, FLAG tagged Wave complex was also found to be inactive when purified in optimal stability conditions, in which case the competing 3× FLAG peptide was still present. Rac in its GTP-bound form and the first SH3 domain of the Nck adapter protein were reported to activate the purified Wave1 complex by a dissociation mechanism [Eden et al., 2002]. However, the addition of the purified GTPase defective mutant RacQ61L was unable to activate our preparation of purified Wave complexes, despite the presence of Wave1 complexes (Fig. 3C). This result is in line with more recent observations that Rac is not sufficient to activate or dissociate the Wave2 complex [Innocenti et al., 2004; Suetsugu et al., 2006]. Similarly, the addition of the first SH3 domain of Nck in fusion with GST did not activate our purified Wave complex significantly, i.e. not more than GST alone that had a very weak effect (Fig. 3D).

These results suggest that Wave proteins are in a conformation, where their acidic Arp2/3 binding site is masked by an interaction within the Wave complex. This interaction is not abolished by Rac or Nck addition. Interestingly, the WCA domain of Wave proteins is predicted to be intrinsically unstructured, unlike most of the rest of the complex (http://iupred.enzim.hu/). Denaturation should not affect the activity of the WCA domain, i.e. its ability to activate the Arp2/3 complex, but should release the WCA domain from its inhibitory interaction. We thus subjected our purified Wave complex to heat (a 10 min incubation at 58°C) and cooled it down to 4°C. This treatment indeed revealed an activity enhancing Arp2/3 mediated actin polymerization (Figs. 3C and 3D). This experiment shows that counterintuitively, Wave activity can be revealed by denaturation of the Wave complex.

To test experimentally the hypothesis that the acidic Arp2/3 binding site of Wave is masked within the complex and can be exposed upon heat treatment, we raised a polyclonal antibody, W2C, targeting the last 15 amino-acids of Wave2 and purified it by affinity for the immunogenic peptide. We then compared this antibody to the previously described W2 antibody targeting an internal peptide of Wave2 in the middle region linking the N-terminal Wave Homology Domain to the C-terminal WCA domain. Both antibodies did efficiently recognize denatured Wave2 in western blots (data not shown). We then used both antibodies to immunoprecipitate purified native Wave complex. The W2 antibody, but not the W2C antibody efficiently immunoprecipitated the Wave complex, suggesting that indeed the C-terminus of the Wave2 molecule is masked in the native Wave complex (Fig. 3E). It should be noted that we used conditions similar to the pyrene actin assay, in that no detergents were used, and this resulted in the immunoprecipitation experiment of a detectable background amount of Wave with non immune IgG beads. When the Wave complex was denatured by the heat treatment and then cooled down, surprisingly, not only the W2C antibody did not immunoprecipitate more Wave2, but also the W2 antibody ceased to immunoprecipitate. In pyrene–actin assays, the W2C blocked the activity of recombinant His-Wave2 (Fig. 3F), as well as the activity revealed by heat denaturation of the Wave complex (Fig. 3G). Note that the block observed with denatured Wave complex cannot be complete, since Wave1 is not recognized by the Wave2 specific W2C antibody. After heat denaturation, the Wave complex subunits were completely pelleted by a 1 h centrifugation at 20,000 × g in a table-top centrifuge (Fig. 3H), This experiment indicates non specific aggregation of the Wave complex subunits after denaturation. This aggregation likely explains why the Wave complex can not be immunoprecipitated even by the W2 antibody after heat treatment. However, the blocking experiment suggest that indeed the activity revealed by this treatment is due to exposure of WCA domains out of agregates.

DISCUSSION

We reported here a fast and efficient purification of the human Wave complex in mild conditions. When assayed in vitro, this material did not activate the Arp2/3 complex, indicating that the acidic Arp2/3 binding site of Wave1 or Wave2 contained in the complex is masked. This conclusion is corroborated by the lack of recognition of the native conformation of Wave2 within its complex by an otherwise blocking antibody targeting the acidic Arp2/3 binding site. This important result is in line with Eden et al. reporting that the Wave1 complex is inactive [Eden et al., 2002] and in opposition with Kim et al. reporting active Wave1 complex at basal state [Kim et al., 2006]. In these experiments using purified endogenous Wave1 complex, denaturation occuring during the many chromatographical steps provides a possible explanation for this discrepancy. Suetsugu et al. reported that the Wave complex immunoprecipitated from cytosol is inactive, in line with our findings that the Wave complex is intrinsically inactive [Suetsugu et al., 2006]. However, in the same study, when the membrane fraction was included in the immunoprecipitation, using detergent for lysis as we do here, these authors found the Wave complex to be weakly but significantly active, in

contrast with ours. Immunoprecipitation or affinity chromatography relies strongly on the precise conditions used in terms of speed or buffer compositions, for the presence of associated factors or posttranslational modifications, as evidenced in this study by the presence of actin asssociated with the Wave complex purified through the PC procedure, but not through the FLAG procedure. Actin might be retrieved in the PC procedure through the WH2 domain of Wave proteins as well as through profilin. The small protein profilin may be at the detection limit of Coomassie in our pattern of purified Wave complex, but may still contribute a large amount of actin through its recruitment by the proline rich regions of Abi and Wave proteins. If the WH2 of Wave proteins contributes this actin, then it implies that the WH2 is exposed at the same time as the nearby acidic domain of the WCA is masked in the native complex. As far as activation is concerned, one can imagine that an activating factor or modification was preserved in the conditions of Suetsugu et al. and lost in our purification protocol, which was designed to assay the basal activity of Wave complexes. To assay endogenously activated Wave complexes, one should probably optimize the conditions required to preserve the specific membrane binding partners of Wave1 and Wave2 such as WRP and IRSp53, respectively [Miki et al., 2000; Soderling et al., 2002]. These proteins belong to the superfamily of BAR/EFC/IMD containing proteins like several important regulators of the WASP family NPFs [Takenawa and Suetsugu, 2007].

Our results indicate that the native Wave complex is inactive. In consequence, this is the state to reach in reconstitution experiments. Only one publication assayed a reconstituted Wave complex obtained after the addition of subunits purified from different expression systems [Innocenti et al., 2004]. This reconstituted Wave2 complex was active, indicating that even though subunits were binding to each other, they did not reach their native conformation. A protocol enabling to reconstitute faithfully a Wave complex thus remains to be designed and may not be straightforward to reach. Indeed, cells assemble the Wave complex using original reactions constituting an assembly pathway that also remains to decipher. We recently provided evidence that the free form of Brk1, a trimer, was used as a precursor in Wave complex assembly, even though a single molecule of Brk1 is complexed to Wave [Derivery et al., 2008]. This implies that along the assembly pathway, the trimeric precursor of Brk1 has to dissociate to incorporate into the nascent Wave complex.

Structural studies require a large amount of homogeneous material. So far only the global architecture of the Wave complex, i.e. the relationships among the five subunits, is known [Gautreau et al., 2004; Innocenti et al., 2004]. Our fast protocol provides an unprecedented amount of Wave complex, several hundreds of microgram. The expression of paralogous subunits in the same cell line creates a combination of Wave complexes. Our system, where one subunit is tagged and specifically retrieved from a cell lysate, enables to simplify this combinatorial complexity. In particular, tagging the Abi subunit, which has several splice variants in addition to three paralogues, enables to homogenize this most variable subunit. Nonetheless, we detected the two paralogues CyFIP1 and CyFIP2 and the two paralogues Wave1 and Wave2 in our preparation, indicating that there is possibly a mixture of four different Wave complexes. Cristallography, which requires milligram amount of a single molecular species, still appears as a long-term goal with this kind of purified Wave complex. However, 3D reconstruction at medium resolution from transmission electron microscopy of purified Wave complex seems within reach in terms of amount and homogeneity given that the mixed Wave complexes are very likely to have the same overall structure. Moreover, we demonstrated here that one can purify Wave complexes tagged on any subunit. Using an additional GFP after the epitope tag, one can envision to locate each subunit extremity by the extra-density in the 3D reconstruction obtained by transmission electron microscopy, as recently achieved in the case of the Arp2/3 complex purified from yeast [Egile et al., 2005].

The molecular replacement of the endogenous subunits by the tagged subunit in these stable cell lines simplifies the repertoire of subunits. This effect, which permitted that an overexpressed single Abi1 splice variant takes over the different endogenous Abi1 and Abi2 variants, is likely due to the instability of subunits when they do not reach their final stable conformation, i.e. within a native Wave complex. That would explain why all subunits were only found in the Wave complex, when the distribution of endogenous subunits was analyzed in a sucrose gradient [Gautreau et al., 2004]. Brk1 was the only exception to this rule. The observation made in a wide variety of organisms that, upon depletion of one subunit by genetic inactivation or RNAi, the others are also depleted [Blagg et al., 2003; Kunda et al., 2003; Innocenti et al., 2004; Steffen et al., 2004; Le et al., 2006; Derivery et al., 2008] supports this idea that to be stable, subunits have to complete the assembly process and become part of the native Wave complex containing all its subunits.

This property of molecular replacement of the endogenous subunits in stable cell lines overexpressing one subunit opens up an easy way to manipulate the repertoire of expressed Wave complexes. One could imagine to reveal specific phenotypes associated with one particular Wave complex and to understand the specific regu-

lation of the Wave complex contributed by a particular subunit. Another potential use of this property is to immunodeplete the Wave complex from a cell-free extract using commercially available antibodies targeting the epitope tag in order to study Wave complex function in biomimetic systems.

CONCLUSIONS

The development of a fast purification protocol for the Wave complex enabled us to purify a large amount of native Wave complex. This material provided an unambiguous answer to the question of its intrinsic activity. We found that the Wave complex is inactive in its basal state. With this ground established, the questions of the factors activating the Wave complex and of the active state of the Wave complex can now be addressed in a simple frame. Moreover, the strategy and protocol we have developped here should be applicable for the purification of other human multiprotein complexes.

ACKNOWLEDGMENTS

We wish to thank Andres Lebensohn and Marc Kirschner for sharing the original observation that the Wave complex is activated upon heat treatment, Lucien Cabanié for help with the Smart System FPLC, Ann Marie Pendergast, Marie-France Carlier, Alan Hall, and Bruce Mayer for providing important reagents, David Cornu and Manuela Argentini from the IMAGIF facility for additional mass spectrometry, and Marie-France Carlier, Beatà Bugyi and Christophe Le Clainche for critical reading of the manuscript.

NOTE ADDED IN PROOF

Before the development of this fast technique to purify the Wave complex, AG previously examined the behaviors of native Wave1 or Wave2 complexes purified by several steps of classical chromatography. These were similar to the one reported here with the tagged complex, which contains a mixture of Wave1 and Wave2. These complexes were intrinsically inactive. They were not activated by GTP bound Rac1, nor by the first SH3 domain of Nck.

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