Assaying WAVE and WASH Complex Constitutive Activities Toward the Arp2/3 Complex

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Contents

Abstract

The Arp2/3 complex generates branched actin networks when activated by Nucleation Promoting Factors (NPFs). Among these, WAVE proteins are required for lamellipodia and ruffle formation, whereas WASH proteins are required for the fission of endosomes. Both WASH and WAVE NPFs are embedded into multiprotein complexes that provide additional functions and regulations. Understanding how these complexes regulate the activity of their NPF starts with the determination of the constitutive activity of the complex. In this chapter, we describe how to efficiently purify the WAVE and WASH complexes

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from human stable cell lines. We also describe how to verify that these complexes are not aggregated, a prerequisite for activity assays. We then provide a protocol to measure their activity toward the Arp2/3 complex using the well-established pyrene actin assay. Finally, we show how our fast purification protocol can be modified to detect the endogenous activity of the WAVE complex, providing an easy readout for the level of WAVE activation in cells.

1. INTRODUCTION

The dynamic actin cytoskeleton is an essential player of cell morphogenesis. Polarized assembly of actin filaments often occurs on membranes, causing their deformation or movement. These actin-based processes are initiated by several classes of actin nucleators (Pollard, 2007). Among these, the Arp2/3 complex nucleates actin filaments from the side of an existing filament, thus generating dendritic arrays. The Arp2/3 complex is constitutively inactive and needs to be activated by Nucleation Promoting Factors (NPFs). The most active NPFs contain a C-terminal domain, called the VCA domain for Verprolin Homology (also known as WH2), Connector and Acidic. The VCA domain binds and activates the Arp2/3 complex (Pollard, 2007). To date, there are four families of VCA containing NPFs, namely WASP, WAVE, WHAMM/JMY, and WASH. WAVE proteins are required for the formation of lamellipodia and membrane ruffles (Takenawa and Suetsugu, 2007). WASP proteins are required for the internalization step of endocytosis, podosome formation, and endosome motility under certain conditions (Benesch *et al.*, 2002, 2005; Jones *et al.*, 2002). WHAMM is required to maintain Golgi morphology and for anterograde membrane transport (Campellone *et al.*, 2008). JMY, despite significant homologies to WHAMM, is involved in transcription and cell migration (Zuchero *et al.*, 2009). Finally, WASH is required for endosome fission (Derivery *et al.*, 2009b).

An interesting property of NPFs is their incorporation into multiprotein complexes. Indeed, WASP proteins have been shown to be in complex with proteins of the WIP family (Derivery and Gautreau, 2010b), WAVE proteins have been shown to be embedded into a complex comprising five subunits namely Sra, Nap, Abi, WAVE, and Brick1 (Eden *et al.*, 2002; Gautreau *et al.*, 2004; Innocenti *et al.*, 2004), and WASH has been recently shown to be part of a complex containing seven subunits namely VPEF, KIAA1033, Strumpellin, WASH, Ccdc53, and the heterodimer of Capping protein (Derivery and Gautreau, 2010a; Derivery *et al.*, 2009b). Interestingly, WASH and WAVE complexes might be structurally analogous (Jia *et al.*, 2010). The incorporation into a multiprotein complex usually provides additional functions and regulations to the NPF. For instance, the WAVE complex provides a molecular link between WAVE proteins and Rac signaling through the Sra

subunit and the WASH complex combines a NPF with an actin barbed end capper. Since both WAVE and WASH proteins in isolation are active toward the Arp2/3 complex (Duleh and Welch, 2010; Eden *et al.*, 2002; Innocenti *et al.*, 2004; Machesky *et al.*, 1999), one might wonder what the intrinsic activity of their respective complex is.

The major bottleneck in the assessment of the activity of multiprotein complexes is their purification. The purification of endogenous multiprotein complexes from cells or tissues usually requires many steps of chromatography, which leads to an overall low yield. For instance, between four and eight steps are required for the purification of WAVE1 and 2 complexes (Eden *et al.*, 2002; Gautreau *et al.*, 2004; Kim *et al.*, 2006; Lebensohn and Kirschner, 2009). This procedure increases the likelihood of denaturation since it lasts several days and use physicochemical conditions that are not necessarily optimal for the stability of the complex.

An alternative to the purification of an endogenous complex is the reconstitution of this complex from recombinant proteins. This method has high yields due to overexpression and has the advantage to simplify the composition of the purified complex. Indeed, cells can express several isoforms for each subunit, giving rise to a combinatorial complexity in native preparations. But this approach is also a long and difficult process. Moreover, such a reconstitution is considered successful only when the reconstituted complex has the same activity as the native one, and thus cannot be used if this constitutive activity is not yet known. The reconstitution of fully recombinant complexes has been obtained for the Arp2/3 (Gournier *et al.*, 2001), the WAVE (Ismail *et al.*, 2009), and the WASH complexes (Jia *et al.*, 2010).

We have developed an intermediate approach between native purifications and fully recombinant reconstitutions. A recombinant tagged subunit is stably expressed in a mammalian cell line and the endogenous complex assembled around this recombinant subunit is purified efficiently by affinity chromatography. Using this strategy, we have greatly improved the yield of the WAVE complex purification over native purification (Derivery *et al.*, 2009a). Importantly, this method can also be used to quickly identify unknown complexes, as we have recently shown for the WASH complex (Derivery *et al.*, 2009b). Using actin polymerization assays, we have shown that the WASH complex purified using this procedure was constitutively active toward the Arp2/3 complex, unlike the WAVE complex (Derivery *et al.*, 2009a,b).

In this chapter, we describe how to quickly and efficiently purify the WASH and WAVE complexes from human stable cell lines. We also provide a protocol to verify that purified complexes are not aggregated, a prerequisite for activity assays. We then describe how to measure their activity toward the Arp2/3 complex using the quantitative pyrene actin assay. Finally, we show how the purification protocol described here can be adapted to detect an endogenous activity of the WAVE complex, which likely reflects its activation in cells.

2. Establishment of Stable Cell Lines Expressing Tagged WAVE and WASH Complexes

We purify WASH and WAVE complexes from cell lines stably expressing one subunit fused to purification tags. In stable cell lines, an interesting property occurs: the exogenous subunit is incorporated into the endogenous complex at the expense of the endogenous one. This property is the result of the degradation of unassembled subunits (Derivery and Gautreau, 2010b; Derivery *et al.*, 2009a). The endogenous complex assembled around this tagged subunit can then be retrieved efficiently by affinity purification. Purification starting from cell lines expressing different subunits can be compared to find the one providing the best incorporation. Actually, we have demonstrated the feasibility of purifying WAVE complexes tagged on any subunit using this system, although with variable efficiency (Derivery *et al.*, 2009a). We will only focus here on the purification of complexes assembled around tagged WAVE and WASH subunits.

To generate these stable cell lines, we use a commercial system in which the plasmid of interest is inserted at a unique well-expressed locus in the host HEK 293 cell line through site directed homologous recombination. As purification tags, we commonly use FLAG and Protein C that are small epitope tags, DYKDDDDK and EDQVDPRLIDGK, respectively. They are recognized by highly specific monoclonal antibodies. FLAG tagged proteins are eluted by competition with an excess of FLAG peptide, whereas PC tagged proteins are eluted by Ca^{2+} chelation since the antibodies requires Ca^{2+} for binding.

2.1. Reagents

pCDNA5 plasmid derived from pCDNA5/FRT/V5-His (Invitrogen) and containing the ORF of human WAVE2 fused to FLAG-HA (M-DYKDDDDK-YPYDVPDYA) or of murine WASH fused to $(His)_6$-PC (M-HHHHHH-EDQVDPRLIDGK) followed by a TEV binding and cleavage site (DYDIPTTENLYFQG). Both tags are fused at the N-terminus and the V5-His tags in the C-terminus are not translated because the natural stop codon of the ORF is kept.

pOG44 plasmid containing the recombinase (Invitrogen).
Flp-In^{TM} T-REx^{TM} 293 cells (Invitrogen).
Hygromycin (Hygrogold, Invivogen).
Growth medium: DMEM supplemented with 10% (v/v) Fetal Calf Serum (FCS) and Penicillin/Streptomycin (0.1 mg/ml each), all reagents from PAA Laboratories.

$2\times$ *HBS*: 274 mM NaCl, 10 mM KCl, 1.5 mM Na$_2$HPO$_4$, 11.1 mM
   D-glucose, 40 mM Na–HEPES, pH 7.05.
XB: 100 mM KCl, 20 mM HEPES, pH 7.7.

2.2. Procedure

250,000 cells are plated in a 3-cm dish. The next day, the medium is replaced by 1.2 ml of fresh medium and cells are transfected using calcium phosphate precipitation procedure 1 h later. The transfection mixture is prepared as follows:

12.4 µl CaCl$_2$ (stock solution at 2 M, filtered)
0.96 µg of pOG44 plasmid
0.2 µg of pCDNA5 plasmid
Sterile Milli-Q water up to 100 µl

This mix is added dropwise into 100 µl of $2\times$ HBS while vortexing at mid power. The resulting solution is then added to cells. Medium is changed after 6–8 h and supplemented with 200 µg/ml hygromycin 24 h later. On the next day, cells are passed into a 10-cm dish and stable transfectants obtained by homologous recombination at the Flp-In site are selected by the continuous presence of 200 µg/ml hygromycin. Clones usually appear after 2 weeks. Stable transfectants are subsequently pooled, amplified, and saved. This homologous recombination procedure is highly efficient and we usually generate many stable cell lines in parallel. A negative control without the pOG44 plasmid is systematically performed to ensure that all the clones obtained are the result of homologous recombination events. These cell lines are quite fragile and we had to use a special protocol to ensure a good viability upon freezing: cells are grown in a 15-cm dish until 80% confluency, trypsinized, and resuspended in 2 ml of 20% growth medium and 80% FCS followed by the addition of 2 ml of 20% DMSO Hybri-Max$^{\text{TM}}$ (Sigma, D2650) and 80% FCS. The resulting solution is aliquoted into four vials, stored at $-80\,^\circ$C for a week, and then transferred into liquid nitrogen for long-term storage. Frozen vials are thawed at 37 $^\circ$C, washed once in growth medium to remove DMSO, and plated in a 10-cm dish. Hygromycin selection is added at 200 µg/ml 2 days later.

The large-scale purification of WAVE and WASH complexes requires a large number of cells, which are amplified in spinner bottles as follows. Cells are amplified to obtain eight 15-cm dishes at 90% confluency and then trypsinized and diluted into 1 l of growth medium supplemented with 100 µg/ml hygromycin in a 2 l spinner flask (Techne, 6027608). The culture is maintained under permanent gentle agitation (50 rpm) on a stirring table (Techne, MCS-104L). Three days later, the culture is split into four spinners and each volume is adjusted to 1 l with growth medium supplemented with

100 μg/ml hygromycin. Volume is adjusted to 1.5 l 2 days later and cells are harvested after two more days. Cells are washed in XB buffer. Cell pellets are quickly frozen in liquid nitrogen and stored at -80 °C. A 6 l culture represents roughly 10^{10} cells and 20 ml of pellet. Flp-In™ T-REx™ 293 cells have retained characteristics of epithelial cells and form spherical hollow structures resembling balloons when cultured in nonadherent conditions. Such structures are not suited for long-term culture and so we usually do not exceed 1 week of culture in spinner bottles. This property also implies that cells must be extensively trypsinized before counting.

3. Large-Scale Purification of WAVE and WASH Complexes

3.1. Reagents

Pellet of cells stably expressing FLAG-HA-WAVE2 of $(His)_6$-PC-WASH (volumes are given for a 10-ml pellet, corresponding to roughly 5×10^9 cells)

WAVE lysis buffer (WLB): 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% DOC, 5% glycerol, 50 mM Na–HEPES, pH 7.7.

WAVE buffer 1 (WB1): 200 mM KCl, 20% glycerol, 50 mM Na–HEPES, pH 7.7.

WAVE buffer 2 (WB2): 400 mM KCl, 20% glycerol, 50 mM Na–HEPES, pH 7.7.

FLAG elution buffer: WB1 supplemented with 0.15 mg/ml 3× FLAG peptide (Sigma F4799). Stock solution at 5 mg/ml in 0.2 M NaCl, 0.1 M Tris–HCl, pH 7.5.

Methylcellulose (Sigma, M0512). Stock solution made at 2% (4000 cP) in Milli-Q water. Let stir overnight at 4 °C to dissolve.

WASH lysis buffer (WHLB): 200 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100, 5% glycerol, 50 mM Na–HEPES, pH 7.4.

WASH buffer 1 (WHB1): 200 mM NaCl, 1 mM $CaCl_2$, 0.02% methyl-cellulose, 50 mM Na–HEPES, pH 7.4.

WASH buffer 2 (WHB2): WHB1 without $CaCl_2$.

PC elution buffer: WHB2 supplemented with 5 mM EGTA.

3.2. Procedure

To minimize degradation, all purification steps are performed at 4 °C. Buffers are prepared the day before the experiment and allowed to equilibrate at 4 °C overnight, along with centrifuge rotors and bottles. The cell pellet is thawed and resuspended in 40 ml of WLB

supplemented with protease inhibitor cocktail (1:500, Sigma, P8340). The solution is rocked for 1 h at 4 °C and cell debris are removed by centrifugation at $3300 \times g$ for 10 min in conical tubes using a swinging bucket rotor (Heraeus, Megafuge 1.0). The supernatant is further clarified by ultracentrifugation at $200,000 \times g$ for 1 h in a fixed-angle rotor (Beckman Type 70 Ti). A thin layer composed of lipids that do not pellet during the high-speed centrifugation is usually observed and carefully removed by pipetting. A small contamination is unavoidable and does not affect subsequent steps. The clarified supernatant is filtered through a 0.22 μm syringe filter unit (Whatman) to avoid subsequent column clogging. The extract is then transferred onto a 25 ml plastic column (Bio-Rad, Econo-Pac) containing 1 ml of FLAG-M2 agarose resin (Sigma, A2220) preequilibrated in WLB buffer. The extract is allowed to recirculate for 4 h at 4 °C with a flow rate of 0.5 ml/min using a peristaltic pump (GE Healthcare, P-1 pump). The column is then washed with 50 ml of WLB buffer, 50 ml of WB1, 50 ml of WB2, and 50 ml of WB1 by gravity flow. The washing step in WB2 constitutes a salt "bump" that enhances purity (WB2 buffer contains twice more KCl than WB1 buffer). FLAG-M2 beads are then transferred into a 2 ml centrifuge tube and several elutions are performed by mixing the resin with 1 ml of FLAG elution buffer. Each elution is 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 $300 \times g$ for 1 min. The WAVE complex usually elutes in the first two fractions which are pooled and allowed to pass through a disposable column to remove remaining beads. The WAVE complex is further concentrated using ultrafiltration (Amicon Ultra-4 3 K, Millipore). The yield of this one-step purification is about 100–200 μg complex starting from 5×10^9 cells. The WAVE complex can be flash frozen in liquid nitrogen and stored at -80 °C. We prefer, however, to use fresh material for activity assays. Figure 33.1 (left panel) shows the pattern of the WAVE complex analyzed by SDS page on 4–12% Bis–Tris gradient gels (Invitrogen).

The WASH complex is purified using the above procedure with the following modifications: WLB is replaced by WHLB, WB1 by WHB1, WB2 by WHB2, FLAG elution buffer by PC elution buffer and FLAG-M2 agarose resin by Protein C affinity resin (Roche, 11815024001), respectively. Since elution from the PC affinity resin (Ca^{2+} chelation) is more efficient than from the FLAG resin, elution is performed by gravity flow at 4 °C. We usually prefer EGTA over EDTA for Ca^{2+} chelation because it does not affect the polymerization of Mg-ATPactin, hence the activity of eluted proteins can be directly tested. The yield of WASH complex purification is usually 5–10 times lower than the one of the WAVE complex. Figure 33.1 (right panel) shows the pattern of the WASH complex analyzed by SDS page on 4–12% Bis–Tris gradient gels.
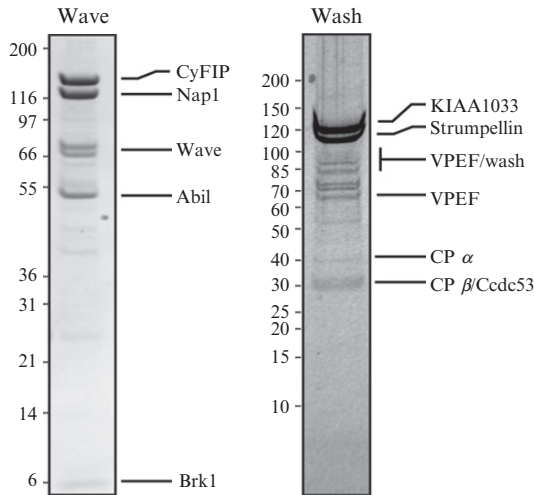
Figure 33.1 Patterns of WAVE and WASH complexes: 10 μg of purified complexes were analyzed by SDS-PAGE and Coomassie blue staining. Identity of the different subunits was determined by mass spectrometry. Molecular weight markers in kDa are indicated on the left of each gel. Left panel shows the pattern of the WAVE complex purified through a tagged Abi1 subunit. Reprinted with permission from Derivery *et al*. (2009a). The WAVE doublet observed here is likely due to phosphorylations and to the presence of two paralogous WAVE genes expressed in 293 cells, WAVE1, and WAVE2. Right panel shows the pattern of the WASH complex obtained using a tagged WASH subunit. Reprinted with permission from Derivery *et al*. (2009b).

4. AGGREGATION ANALYSIS OF WAVE AND WASH MULTIPROTEIN COMPLEXES

As with any protein, care must be taken concerning aggregation of multiprotein complexes when one wants to perform activity assays. For instance, an intramolecular inhibition can be released upon aggregation, leading to activity artifacts. This is especially the case for multiprotein complexes, which are bigger, so have larger contact interfaces, and whose purification yields are usually low, leading to diluted solutions. The WAVE complex offers a textbook example to illustrate this point. Depending on purifications, the native WAVE complex has been found active (Kim *et al*., 2006) or not (Derivery *et al*., 2009a; Eden *et al*., 2002; Ismail *et al*., 2009; Lebensohn and Kirschner, 2009). It was recently shown that denaturation by heat (Derivery *et al*., 2009a; Lebensohn and Kirschner, 2009) or freezing in the absence of cryoprotectant (Ismail *et al*., 2009) leads to the detection of some activity of the complex. This likely explains the results of Kim and colleagues and illustrates how important it is to find optimal buffer conditions for the stability of a new complex before starting activity assays.

Several methods are available to study the aggregation state of proteins, such as DLS and analytical gel filtration. These methods, however, require a large amount of material and thus cannot be practically used to study the aggregation of multiprotein complexes, whose purification yields are low. We describe here a quick and efficient method to analyze the aggregation state of WASH and WAVE complexes based on controlled ultracentrifugation experiments. This method requires only small amounts of starting material and thus can be performed on small–scale purifications, making it particularly suited for buffer screening. Unfortunately, there is no universal buffer and every complex is likely to require such buffer optimization. For instance, the WASH complex is not stable in the conditions optimized for the WAVE complex.

4.1. Procedure

The aggregation assay described here requires only limited amounts of starting material; so, it is performed with complexes retrieved in small-scale purifications. Small-scale purification of WASH or WAVE complexes is performed by scaling down the above described, large-scale protocol. We start from two 15-cm dishes of cells (6×10^7 cells) lysed in 1 ml of lysis buffer. Clarification of the lysate is performed by a single spin at $20,000 \times g$ for 10 min at 4 °C in a tabletop centrifuge (Eppendorf, model 5424). We use only 20 μl of affinity resin and all washes are performed with 1 ml of buffer. For elution, the resin is resuspended in 20 μl of elution buffer and allowed to sit overnight at 4 °C. The next day, the slurry is centrifuged at $300 \times g$ for 1 min and the supernatant carefully pipetted. 20 μl of fresh elution buffer is added to the beads and after centrifugation of the slurry at $300 \times g$ for 1 min, the supernatant carefully pipetted and pooled with the previous one. Remaining beads are removed by two sequential spins at $20,000 \times g$ for 30 s.

20 μl of eluate is spun in a Beckman TLA100 rotor at 40,000 rpm for 30 min at 4 °C in a 7×20 mm polycarbonate tube (Beckman, 343775). 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 WASH complex, whose sedimentation coefficient is 11S (Gautreau *et al.*, 2004). The WASH complex, with a sedimentation coefficient of 12.5S (Derivery *et al.*, 2009b) requires 1.95 h to pellet. Thus the pellet after a 30 min spin contains only aggregated complexes. The supernatant is carefully pipetted and the pellet is resuspended in 20 μl of boiling 1× SDS–PAGE loading buffer (diluted with fresh elution buffer). Equal volumes of eluate, supernatant, and resuspended pellet are then analyzed by SDS–PAGE followed by Coomassie blue staining or Western blotting, depending on complex abundance.

Figure 33.2A illustrates how increasing the KCl concentration from 100 to 200 mM decreases the aggregation of the WAVE complex. Exact buffer
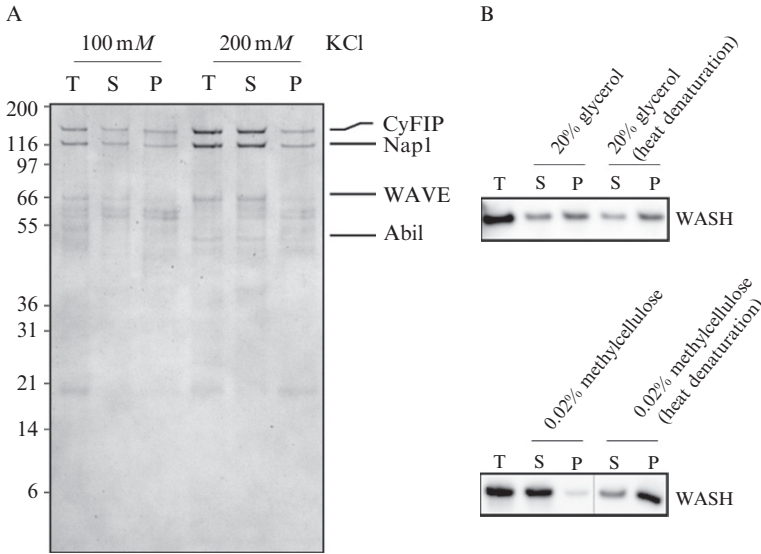
Figure 33.2 Screen for stability conditions of WAVE and WASH complexes. Small-scale purifications of WAVE and WASH complexes were performed in indicated buffer. WAVE and WASH complexes were purified using tagged WAVE and WASH subunits, respectively. Purified material was then subjected to ultracentrifugation and both supernatant and pellet were analyzed by SDS-PAGE. T: total, S: supernatant, P: pellet (aggregated material). Comparison of the amount of material between total and supernatant indicates the level of aggregation. (A) Coomassie blue stained gel showing that increasing the KCl concentration from 100 to 200 mM through the entire purification decreases the aggregation of the WAVE complex. (B) Western blot showing that replacement of 20% glycerol by 0.02% methylcellulose in wash buffers decreases the aggregation of the WASH complex. Heat denaturation (95 °C for 5 min) is used here as a positive control of aggregation.

compositions in this experiment are as follows (buffer names refer to the large-scale purification scheme):

Sample	100 mM KCl	200 mM KCl
WLB	150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% DOC, 5% glycerol, 50 mM Na–HEPES, pH 7.7.	
WB1 = WB2	20% glycerol, 50 mM Na–HEPES, pH 7.7	
	100 mM KCl	200 mM KCl

Figure 33.2B illustrates how the substitution of 20% glycerol with 0.02% methylcellulose decreases the aggregation of the WASH complex. Heat denaturation (95 °C for 5 min) is used as a positive control of aggregation. Exact buffer compositions in this experiment are as follows:

Sample	20% glycerol	0.02% methylcellulose
WHLB	200 mM NaCl, 1 mM CaCl$_2$, 1% Triton X-100, 5% glycerol, 50 mM Na–HEPES, pH 7.4.	
WHB1	200 mM NaCl, 1 mM CaCl$_2$, 50 mM Na–HEPES, pH 7.4	
	20% glycerol	0.02% methylcellulose
WHB2	200 mM NaCl, 50 mM Na–HEPES, pH 7.4	
	20% glycerol	0.02% methylcellulose

To find optimal buffer conditions, we commonly vary salt concentration (NaCl or KCl: 50–400 mM) and additives (glycerol: 0–30%, sucrose 0–0.4 M or methylcellulose 0–0.1%). We usually keep the same salt concentration through the entire purification and add the additives only in the wash buffers. Using this assay, we found that the stability of the WAVE complex requires a KCl concentration between 200 and 400 mM in addition to glycerol (between 15% and 20%). On the other hand, the stability of the WASH complex requires a KCl concentration of 200 mM in addition to 0.02% methylcellulose.

5. ACTIVITY MEASUREMENTS USING PYRENE ACTIN POLYMERIZATION ASSAYS

Once complexes are purified and in an unaggregated state, their constitutive activity toward the Arp2/3 complex can be assayed. To monitor the kinetics of Arp2/3 mediated actin polymerization, we use the robust and quantitative pyrene actin assay, in which actin polymerization is followed in a fluorimeter through the incorporation of a fluorescent tracer, pyrene actin. The fluorescence intensity of pyrene actin increases by an ~25-fold upon polymerization (Kouyama and Mihashi, 1981), allowing quantitative analysis of polymerization kinetics. Alternatively, a microscopy-based assay of Arp2/3 activity can be used (Blanchoin et al., 2000). This assay requires less material but its quantification is less convenient. Hence, this assay will not be described here.

5.1. Reagents

G buffer: 0.2 mM ATP, 0.1 mM CaCl$_2$, 0.1 mM DTT, 0.01% NaN$_3$, and 5 mM Tris–HCl, pH 8.0.

10× KMEI: 500 mM KCl, 10 mM MgCl$_2$, 10 mM EGTA, and 100 mM Imidazole–HCl, pH 7.0.

10× Exchange buffer: 2 mM EGTA and 0.2 mM MgCl$_2$.

Rabbit muscle actin, isolated from acetone powder as described (Spudich and Watt, 1971) and stored in G buffer. Alternatively, this reagent can be purchased from Cytoskeleton, Inc.

Pyrene actin, prepared as described in a previous volume of *Methods in Enzymology* (Zigmond, 2000) and stored in G buffer. Alternatively, this reagent can also be purchased from Cytoskeleton, Inc.

Arp2/3 complex, purified as described (Egile *et al.*, 1999) or purchased from Cytoskeleton, Inc.

5.2. Procedure

To minimize concentration errors due to pipetting, a common stock solution of actin/pyrene actin (90/10%) at 12 μM in G buffer is prepared and kept on ice. A typical actin polymerization assay is made as follows:

- MgATP-G-actin is first prepared by mixing 37.5 μl of the actin/pyrene actin stock (12 μM) with 4.5 μl of 10× Exchange buffer and 3 μl of G buffer. This solution is incubated on ice for 5 min and the resulting MgATP-G-actin (at 10 μM) is used within 1 h.
- The polymerization mixture is then prepared at room temperature by adding components in this order:

| Volume added	Final concentration
16 μl of 10× KMEI	1×
87.5 μl G buffer	
1.5 μl Arp 2/3 complex (stock at 4.5 μM)	43 nM
15 μl WAVE or WASH complex	20–200 nM
40 μl of MgATP-G-actin (stock at 10 μM)	2.5 μM (10% pyrene)
160 μl total	

It is important to add the G buffer before fragile components like the Arp2/3, WASH, or WAVE complexes so that the high concentration of KCl of the 10× KMEI is diluted. We do not recommend increasing too much the volume of WAVE/WASH complex since additives such as glycerol and sucrose affect actin polymerization. Low activities are often hidden by the spontaneous nucleation of actin, which can be reduced by diminishing the actin concentration in the assay down to 1 μM.

- The reaction is mixed, transferred immediately to a quartz cuvette, and fluorescence is monitored in a SAFAS Xenius fluorimeter (Safas SA, Monaco), where up to 10 kinetics can be followed in parallel. Pyrene fluorescence is measured at 407 nm with excitation at 365 nm.

One must be extremely careful about controls when observing an activity with material retrieved with only one step of purification, since an increase of the actin polymerization rate can arise from many sources other than the constitutive activity of the complex assayed. We recommend the following controls:

- Assaying the activity of an equal amount of buffer without complex since additives can affect actin polymerization.
- Removing the Arp2/3 complex from the polymerization mix, since other actin nucleators, like formins, might copurify, as described for the WAVE complex (Beli *et al.*, 2008; Yang *et al.*, 2007). A "nucleation" effect might also be observed if F–actin stabilizing proteins or filament cappers are present in the preparation.
- Assaying the activity of material purified from a cell line expressing the empty vector, or changing the purification tags, to exclude a possible contamination from the affinity resin.
- Using blocking antibodies targeting the supposedly active part of the complex, if available, to ascribe the observed activity to a subunit in particular.
- Assaying the activity of complexes purified through tags present on different subunits. It is indeed reasonable to think that the measured activity might be due to a contamination by a small pool of unassembled, unregulated subunit, since the purification protocol described here only relies on the tag present on the exogenous subunit. This is especially true when the tagged subunit is the one harboring the activity, like WASH or WAVE. Fortunately, in the stable cell line context described here, we observed that the excess of unassembled exogenous subunit is usually degraded (Derivery *et al.*, 2009a). Along with this idea, we could not detect any differences in the constitutive activity of WAVE complexes tagged on either WAVE2 or Abi1 (Derivery *et al.*, 2009a).

Constitutive activities of WAVE and WASH complexes are displayed in Fig 33.3. Figure 33.3A shows that the WAVE complex is constitutively inactive since no increase of the polymerization rate is observed when it is added to Actin and the Arp2/3 complex. The WAVE complex has the counterintuitive property to reveal its activity upon heat denaturation (Derivery *et al.*, 2009a; Lebensohn and Kirschner, 2009), providing a useful internal positive control in the experiment. On the other hand, Fig. 33.3B shows that the WASH complex is constitutively active. Indeed, addition of WASH complex highly increases the polymerization rate of actin in the presence of the Arp2/3 complex. This phenomenon is specifically due to the activation of the Arp2/3 complex by the VCA domain of WASH, since a blocking antibody targeting the VCA domain can alleviate this increase of the polymerization rate. Interestingly, in the absence of Arp2/3 complex,
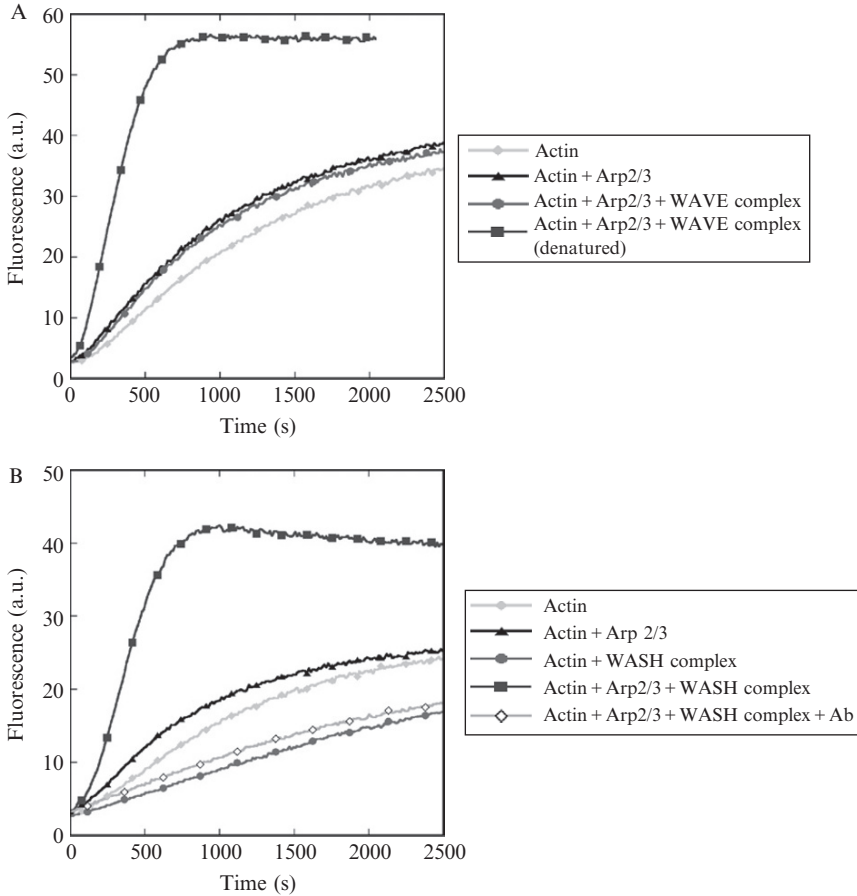
Figure 33.3 Constitutive activity of WAVE and WASH complexes. WAVE or WASH complex activities toward purified Arp2/3 complex was monitored by pyrene fluorescence of Mg-ATP actin. WAVE and WASH complexes were purified using tagged WAVE and WASH subunits, respectively. Conditions: 10 mM imidazole–HCl, 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. $\sim$20 nM WAVE complex or 35 nM WASH complex was added to 43 nM Arp2/3 complex and 2.5 μM actin (10% pyrene labeled). WASH-VCA blocking antibody was added at 40 nM. (A) The WAVE complex is constitutively inactive. Heat denaturation (58 °C for 10 min), which reveals the activity of the WAVE complex, is shown as a positive control. The purified WAVE complex used in this experiment came from a small-scale purification, but no difference in terms of activity is observed in purification performed at small or large scale. (B) The WASH complex is constitutively active. Reprinted with permission from Derivery *et al.* (2009b). The complex used in this experiment was purified as described above, except that methylcellulose (0.02%) was substituted by sucrose (0.4 M). Substituting sucrose by methylcellulose does not affect the activity of the complex but prevents its denaturation upon flash freezing in liquid nitrogen.

the WASH complex slowed down the kinetics of spontaneous actin polymerization, a property attributed to a low barbed end capping activity of the complex (Derivery *et al.*, 2009b).

6. Detection of an Endogenous Activity of the WAVE Complex

The WAVE complex is thought to alternate between a cytosolic, inactive conformation and a membrane-bound, active one (Suetsugu *et al.*, 2006). Although we recover both pools with our purification method, we do not detect any activity of the WAVE complex. This suggests that the transient interactors responsible for the activity of the membrane pool are washed away during the purification. It is indeed intuitive to distinguish the weak interactions formed between the WAVE complex and its activator(s) in response to signaling from the strong interactions between the subunits maintaining complex cohesion. If one manages to preserve such weak interactions, the detection of the endogenous activity of the WAVE complex should be possible. Having a quantitative readout of the activity level of the WAVE complex in cells is useful to decipher the signaling pathways that activate the WAVE complex. For instance, this assay can be combined with siRNA or drug treatments. We will now describe how the purification protocol described here can be modified to detect the endogenous activity of the WAVE complex.

Transient interactors are lost during the purification mainly for two reasons. First, if handling times are long, the system can reach equilibrium during washing steps, leading to dissociation due to the law of mass action. The only way to overcome this phenomenon is to develop the fastest purification protocol possible. The second phenomenon is that the salts contained in washing buffers compete with the partners for binding to the complex of interest. This is especially true in case of weak interactors, which have a lower number of contacts. To overcome this phenomenon and preserve such interactions, one can think to screen milder buffer conditions. Buffer modifications must, of course, be performed according to stability conditions. The small-scale purification protocol of the WAVE complex described here fulfills both the speed requirement and the possibility to screen various buffers, since handling time is short and that it requires only a limited amount of starting material. In addition, it is compatible with activity measurements (Fig. 33.3A).

Figure 33.4 shows that an endogenous activity of the WAVE complex can be detected if the high salt wash (WB2 buffer) is omitted during the purification (compare "high salt" and "low salt" curves). This activity depends on the Arp2/3 complex. Thus, this activity cannot be accounted
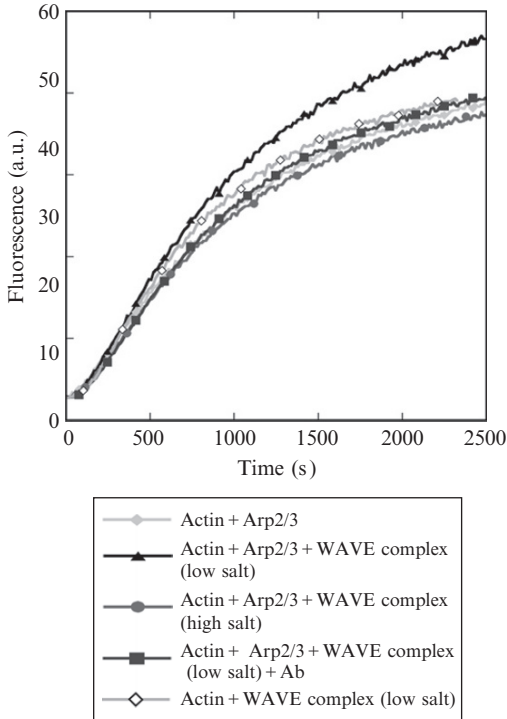
Figure 33.4   Detection of an endogenous activity of the WAVE complex. Two small-scale purifications of the WAVE complex were performed by tagging the WAVE subunit. The washing step at high concentration of KCl (WB2 buffer) was omitted in one sample, referred to as "low salt." WAVE complex activity in both samples was monitored by pyrene fluorescence using conditions identical to those of Fig. 33.3. Interestingly, an activity is detected in the "low salt" sample. This activity is specific of the WAVE–Arp2/3 pathway since removing the Arp2/3 complex or adding a WAVE-VCA blocking antibody (100 nM) can alleviate it. This activity is thus due to a transient activator of the WAVE complex, which is lost during the high salt wash.

for by the formin mDia1, another actin nucleator previously shown to interact with the Abi subunit of the WAVE complex (Beli *et al.*, 2008; Yang *et al.*, 2007). Moreover, this activity depends on WAVE, since it is lost when a blocking antibody specifically targeting the VCA domain of WAVE is added. This control establishes that this activity cannot be accounted for by N-WASP, another NPF previously shown to interact with Abi (Innocenti *et al.*, 2005). This experiment shows that an activity of the WAVE complex can be detected and that an uncharacterized activator weakly associated to the WAVE complex is responsible for it. This activity likely reflects the level of WAVE complex activation in cells.

7. Concluding Remarks

In this chapter, we have described a method to purify and address the constitutive activity of WAVE and WASH complexes. Importantly, this method can be used to identify novel multiprotein complexes and should thus be applied to other NPFs for which the embedment into multiprotein complex is still unknown, like JMY or WHAMM.

It is quite intriguing to find the WASH complex constitutively active. It is indeed unlikely that NPFs are unregulated in cells, given the major cytoskeleton rearrangements they induce. A WAVE-like model in which the NPF is maintained inactive through its incorporation within a regulatory complex that connects it to signaling pathways is more satisfactory. As illustrated for the case of the WAVE complex, it is possible that the one-step purification protocol described here preserved interactions with unknown activator(s). Alternatively, several posttranslational modifications essential for activity might have been preserved, like phosphorylations, as shown for the Arp2/3 and WAVE complexes (Lebensohn and Kirschner, 2009; LeClaire et al., 2008). This might explain why a longer, four-step purification of a recombinant WASH complex using coinfection with multiple baculoviruses yielded an inactive complex (Jia et al., 2010). To understand this discrepancy between purifications, one can notice that, like the WAVE complex, the WASH complex is present into two pools in cells. One pool is cytosolic (Duleh and Welch, 2010), whereas the other is bound to endosomal membranes, where it colocalizes with actin and the Arp2/3 complex (Derivery et al., 2009b). The cytoplasmic pool is likely recruited onto endosomes upon signaling and must reflect the true constitutive activity of the complex. It would be thus interesting to address the activity of the WASH complex purified from cytosolic extracts using the protocol described here. We expect it to be inactive, as shown for the WAVE complex (Suetsugu et al., 2006).

We believe that this purification method based on stable cell line provides a tremendous number of possibilities to dissect the activity of virtually any mammalian complexes. For instance, one can imagine addressing the activity of mutant complexes by introducing point mutations in the tagged subunit, provided that the mutation does not impair cell growth. Another exciting potentiality of the system would be to address the activity of complexes lacking regulatory subunits by purifying material from siRNA-treated cells.

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