References

Chamberlain, C., and Hahn, K. M. (2000). Watching proteins in the wild: Fluorescence methods to study protein dynamics in living cells. *Traffic* **1,** 755–762.

Chamberlain, C. E., Kraynov, V., and Hahn, K. M. (2000). Imaging spatiotemporal dynamics of Rac activation *in vivo* with FLAIR. *Methods Enzymol.* **325,** 389–400.

Danuser, G. (1999). Photogrammetric calibration of a stereo light microscope. *J. Microscopy* **193,** 62–83.

Del Pozo, M. A., Kiosses, W. B., Alderson, N. B., Meller, N., Hahn, K. M., and Schwartz, M. A. (2002). Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. *Nat. Cell Biol.* **4,** 232–239.

Nalbant, P., Hodgson, L., Kraynov, V., Toutchkine, A., and Hahn, K. M. (2004). Activation of endogenous Cdc42 visualized in living cells. *Science* **305,** 1615–1619.

Patterson, G. H., and Piston, D. W. (2000). Photobleaching in two-photon excitation microscopy. *Biophys. J.* **78,** 2159–2162.

Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* **18,** 578–585.

Shen, F., Hodgson, L., Rabinovich, A., Pertz, O., Hahn, K. M., and Price, J. H. (2004). Functional proteometrics for cell migration. *Cytometry.* In press.

Toutchkine, A., Kraynov, V., and Hahn, K. (2003). Solvent-sensitive dyes to report protein conformational changes in living cells. *J. Am. Chem. Soc.* **125,** 4132–4145.

[13] Cdc42 and PI(4,5)P_2-Induced Actin Assembly in *Xenopus* Egg Extracts

By ANDRES M. LEBENSOHN, LE MA, HSIN-YI HENRY HO, and MARC W. KIRSCHNER

Abstract

Xenopus egg cytoplasmic extracts have been used to study a variety of complex cellular processes. Given their amenability to biochemical manipulation and physiological balance of regulatory proteins, these extracts are an ideal system to dissect signal transduction pathways leading to actin assembly. We have developed methods to study Cdc42 and PI(4,5)P_2-induced actin assembly in *Xenopus* egg extracts. In this chapter, we describe detailed procedures to prepare *Xenopus* egg extracts, Cdc42, and PI(4,5)P_2 for use in actin assembly experiments. We also describe a fluorometric pyrene actin assay for quantitative kinetic analysis of actin polymerization and a microscopic rhodamine actin assay for quick measurement of actin rearrangements in extracts. Finally we provide a protocol for immunodepletion of proteins and discuss the use of immunodepletion

METHODS IN ENZYMOLOGY, VOL. 406 0076-6879/06 $35.00

DOI: 10.1016/S0076-6879(06)06013-7

and rescue experiments for functional analysis of components in the extracts.

Introduction

Cytoplasmic extracts prepared from the eggs of the African clawed frog *Xenopus laevis* have proven to be a powerful cell-free system for dissecting a variety of complex biological processes, including nuclear assembly and disassembly (Newport and Spann, 1987), chromosome condensation (Ohsumi *et al*., 1993), spindle assembly (Sawin *et al*., 1992), DNA replication (Hutchison and Kill, 1989), and the cell cycle (Murray and Kirschner, 1989). A unique property of *Xenopus* egg extracts is that they recapitulate these complex processes in a manner very similar to that observed in intact cells. This is because they are prepared at high protein concentrations and maintain a physiological balance of regulatory proteins. Yet, unlike intact cells or whole organisms, extracts are amenable to extensive biochemical manipulation. Proteins can be removed by immunodepletion and added back at known concentrations or in mutant forms, and the extracts can be fractionated to purify components required for an activity of interest.

The utility of *Xenopus* egg cytoplasmic extracts for studying actin assembly was first established by experiments demonstrating that the intracellular pathogen *Listeria monocytogenes* moved in extracts propelled by actin comet tails very similar to those formed in infected somatic cells (Marchand *et al*., 1995; Theriot *et al*., 1994). These results suggested that the extracts contained the machinery required for actin assembly. Our own experiments then demonstrated that extracts also contained the components required to couple this machinery to cellular signaling intermediates such as phosphoinositides and Cdc42 (Ma *et al*., 1998a), making them an ideal system to dissect signal transduction pathways leading to actin assembly.

Our initial studies showed that Cdc42 was required for phosphatidylinositol-4,5-biphosphate (PI[4,5]P_2)-induced actin assembly (Ma *et al*., 1998a). Then, using Cdc42 as the agonist and microscopic and kinetic assays as activity readouts, we fractionated *Xenopus* egg extracts to purify downstream components required to modulate actin assembly. This strategy, combined with immunodepletion experiments, led us to discover that the Arp2/3 complex and N-WASP mediate actin polymerization downstream of Cdc42 (Ma *et al*., 1998b; Rohatgi *et al*., 1999), demonstrating for the first time that the actin nucleating activity of the Arp2/3 complex can be modulated by a cellular signaling pathway. The finding that N-WASP directly links Cdc42 to the Arp2/3 complex also helped establish the role of WASP family proteins as important nodes connecting upstream

signals to actin nucleation. Furthermore, purification from *Xenopus* egg extracts revealed that the native N-WASP protein is almost entirely in a complex with WIP (WASP interacting protein) and led to the identification of Toca-1 (transducer of Cdc42-dependent actin assembly) as an essential modulator of this complex (Ho *et al*., 2004). Unlike recombinant N-WASP, which can be activated by Cdc42 alone, activation of the N-WASP-WIP complex requires both Cdc42 and Toca-1.

The results of these studies, along with the work from a number of other laboratories, delineated a pathway from PI(4,5)P_2 through Cdc42, Toca-1, and the N-WASP-WIP complex regulating actin nucleation by the Arp2/3 complex. On the basis of these findings, we have been able to reconstitute Cdc42-mediated actin assembly *in vitro* using purified components (Ho *et al*., 2004; Rohatgi *et al*., 1999), a system that is the topic of the following chapter.

In this chapter, we describe how to prepare *Xenopus* egg extracts optimized for actin assembly experiments and how to prepare and use the agonists Cdc42 and PI(4,5)P_2 to stimulate actin polymerization. We also describe two assays to measure actin assembly in extracts: a fluorometric pyrene actin assay for quantitative kinetic analysis of polymerization reactions and a microscopic rhodamine actin assay for quick measurement of actin assembly. Finally, we provide a protocol for immunodepletion of proteins and discuss the use of immunodepletion and rescue experiments for functional analysis of components in the extract.

Preparation of *Xenopus* Egg Extracts for Actin Assembly Experiments

The procedure for preparing cycling and cytostatic factor (CSF)-arrested extracts used in cell cycle studies has been described in great detail (Murray, 1991). Here we will describe a modified protocol for making CSF-arrested extracts optimized for use in actin assembly experiments.

We typically obtain between 1 and 2 ml of concentrated high speed supernatant (HSS) per frog. Because we only use 20 μl of HSS per kinetic assay reaction and 4 μl of HSS per microscopic assay reaction, preparations starting with five frogs allow for relative ease of handling and yield enough material for a few hundred reactions. However, we have successfully prepared extracts starting with as few as one frog and as many as 200. We only use high-quality eggs (described later) to prepare extracts, and depending on the season, the age of the frogs, the water temperature, and a number of other factors, the quality of the eggs can vary significantly. Summer is usually the worst season, older (larger) frogs tend to do

poorer than young ones, and water temperatures above 20° seem to be detrimental. Because of this variability, it is advisable to start preparations with a few extra frogs, given that generally some will lay poor-quality eggs that are useless. Except during the summer months, typically ~70% of the frogs lay good-quality eggs. Because the quality of the extracts decreases with prolonged handling time, careful planning and advanced preparation of reagents and instruments is also crucial.

Reagents

Pregnant mare serum gonadotropin (PMSG, Calbiochem 367222): 100 U/ml prepared in sterile water, stored in aliquots at −20° and warmed up to room temperature before use. Make 0.5 ml per frog.

Human chorionic gonadotropin (HCG, Sigma CG-10): 1000 U/ml prepared in sterile water, stored in aliquots at −20° and warmed up to room temperature before use. Make 0.5 ml per frog.

Marc's modified Ringers' (MMR): 100 m*M* NaCl, 2 m*M* KCl, 1 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 0.1 m*M* EDTA, 5 m*M* Na-HEPES, pH 7.8, at 16°. Prepared from a 25× stock. Make 2 l per frog for egg laying, plus 1 l per frog for washes.

Dejellying solution: MMR + 2% w/v cysteine, pH 7.8, at 16°. Prepared within 1 h of use from 25× MMR and solid L-cysteine (Sigma C-7352), and titrated to pH 7.8 with NaOH. Make 240 ml per frog.

Extract buffer (XB): 100 m*M* KCl, 0.1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* K-HEPES, pH 7.7, at 16°, 50 m*M* sucrose. Make 400 ml per frog.

Extract buffer for CSF extracts (CSF-XB): 100 m*M* KCl, 2 m*M* $MgCl_2$, 10 m*M* K-HEPES, pH 7.7, at 16°, 50 m*M* sucrose, 5 m*M* EGTA. Make 200 ml per frog for washes, plus 20 ml per frog for dilution of extract.

Protease Inhibitors (PIs): 10 mg/ml each leupeptin (Sigma L-2884), pepstatin (Sigma P-5318), and chymostatin (Sigma C-7268) prepared in DMSO, stored in aliquots at −20° and diluted into the buffer immediately before use. Make 32 μl per frog.

Energy mix for CSF extracts (20× CSF-Energy mix): 150 m*M* phosphocreatine (Sigma P-7936), 20 m*M* ATP (adjusted to pH 7.0 with Tris base), 20 m*M* $MgCl_2$, stored in aliquots at −20°. Make 200 μl per frog.

Priming Frogs and Inducing Ovulation

Frogs are kept at 16° in dechlorinated water (preferably treated by reverse osmosis [RO], but tap water can also be used) made 15 m*M* in NaCl (preferably supplemented with 2% w/w Instant Ocean synthetic sea salts [Aquarium Systems, Inc.], which provide trace elements). On day 1,

female frogs are primed for ovulation by injecting 50 units of pregnant mare serum gonadotropin (PMSG) into the dorsal lymph sac using a 27-gauge ½-inch sterile needle. After priming with PMSG, frogs are not fed until after the eggs have been collected to prevent excrement and regurgitated food from ruining the eggs. Ovulation is induced on any day from day 5 to at least day 12 by injecting 500 units of human chorionic gonadotropin (HCG) as described before. Before they begin to lay eggs, frogs are individually transferred to separate containers containing 2 l of Marc's modified Ringers' (MMR), and they are covered with perforated lids to allow air flow. The time from HCG injection to the beginning of egg laying is somewhat variable, but averages 12–14 h at 16°. Therefore, we usually inject the frogs around noon and transfer them in the evening approximately 8 h after injection, so that all the eggs are laid in MMR, but the period during which the frogs are kept in the same container as the eggs is minimized. On the day that we induce ovulation, we also prepare and refrigerate all buffers required for making the extract on the following day (except for dejellying solution, which must be prepared within 1 h of use).

Collecting Eggs

Eggs are collected ~20 h after HCG injection (ideally no later than 10 h after they have been laid). Primed frogs typically lay packed egg volumes between 20 and 40 ml (measured after the eggs have settled). The frogs are removed from the containers, most of the MMR is poured off, and the eggs are inspected visually to assess their quality. Good-quality eggs are round, uniform in appearance and size, and are detached from one another (Fig. 1A). Poor-quality eggs are often attached together, forming strings or clumps, may have irregular shapes, may appear large and pale, or may exhibit extensive pigment mottling or variegation (Fig. 1B). Any batch in which more than 10% of the eggs are of poor quality is discarded. A few poor-quality eggs can be removed individually from good batches by picking them out with a glass Pasteur pipette whose tip has been broken off to give an orifice about 4 mm in diameter and fire polished. All batches of good-quality eggs are pooled together and washed five times with MMR (~5 packed egg volumes each wash) to remove shed frog skin, excrement, and other detritus. Because the eggs settle quickly to the bottom of the container, washes are carried out by pouring solution into the container and decanting carefully, but swiftly, while eggs are resettling at the bottom, but the debris is still floating. For a five-frog prep, collection, sorting, and washing should take no longer than 45 min.

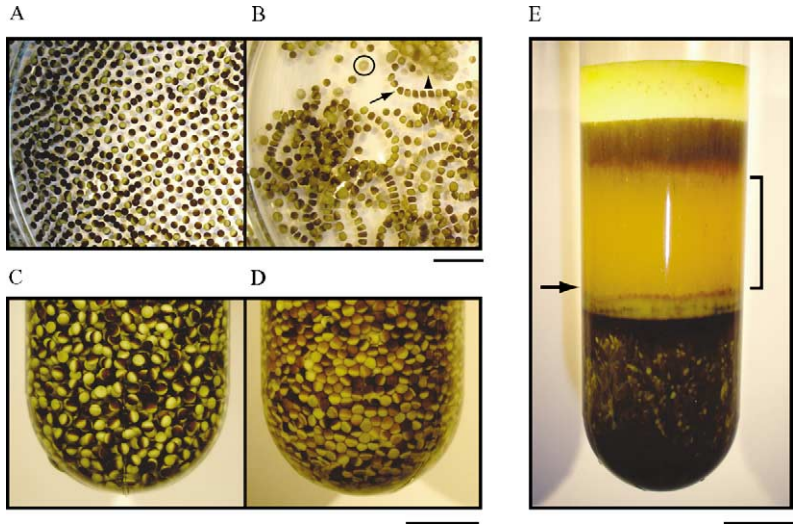

FIG. 1. *Xenopus laevis* eggs. (A) Good-quality eggs are round and uniform. (B) Poor-quality eggs are often attached together forming strings (arrow) or clusters (arrowhead) or may be large and pale (circled). (C) Before dejellying, eggs are separated by their transparent jelly coats. (D) Dejellied eggs pack as tight spheres without any visible separation. (E) After centrifugation, three major layers result: lipid, cytoplasm, and yolk going from top to bottom. Cytoplasmic extract (marked by the bracket) is collected by puncturing the side of the tube near the bottom of the middle layer (at the site indicated by the arrow). Scale bar = 10 mm.

Making Cytoplasmic Extract

As much MMR as possible is poured off. Eggs are dejellied by adding ~4 volumes of dejellying solution and swirling gently at short intervals for 5–10 min, preferably keeping the temperature as close to 16° as possible. The supernatant is decanted carefully, and the eggs are rinsed for another 2–5 min with ~half the original volume of dejellying solution. Dejellying should not be allowed to proceed longer than necessary, so during this step eggs should be monitored closely. Dejellying is complete when eggs pack as tight spheres without any visible separation caused by their jelly coats (compare Fig. 1C and 1D), and the packed egg volume is reduced to 20–25% of the original volume.

Dejellied eggs are washed four times in extract buffer (XB, ~10 packed dejelied egg volumes each wash) and twice in extract buffer for CSF extracts (CSF-XB, ~10 volumes each wash). Because dejellied eggs are very fragile and prone to lysing spontaneously, these washes should be carried out as quickly as possible. Damaged eggs turn pale and tend to float

above intact eggs, so they can be gently poured off after each wash or manually discarded using a wide-mouthed polished Pasteur pipette.

For the subsequent centrifugation steps, any soft-walled centrifuge tube allowing perforation with a needle and suitable for a swinging-bucket rotor able to reach 25,000*g* can be used. We routinely use Beckman Thinwall Polyallomer or Ultra-Clear 13- × 51-mm tubes for spinning dejellied egg volumes up to 5 ml in SW 50.1 or MLS 50 rotors, 14- × 95-mm tubes for spinning volumes up to 12 ml in an SW 40 Ti rotor, and 25- × 89-mm tubes for spinning volumes up to 36 ml in an SW 28 rotor. Before transferring the eggs, each centrifuge tube is loaded with a volume of CSF-XB plus 100 μg/ml (each) protease inhibitors (PIs) equivalent to ~10% of the volume of eggs that will be transferred. After the final wash of the eggs with CSF-XB, as much buffer as possible is poured off, and the eggs are carefully transferred until each centrifuge tube is full. To avoid damaging the eggs, a wide-mouthed fire polished Pasteur pipette can be used to transfer small volumes, or a Falcon 100 ml serological pipette (BD Biosciences 357600) can be used to transfer large volumes (this pipette has a wide opening and smooth internal edges). After transferring the eggs, excess CSF-XB is removed, eggs are spun at 300*g* for 1 min to displace interstitial buffer trapped between them, and the excess buffer from the top of the tube is removed again, thereby decreasing the dilution of the cytoplasm in the final extract. Eggs are then crushed by spinning at 25,000*g* for 20 min at 4°. After this step, all handling is done at 4°, and extracts are kept on ice.

After centrifugation, eggs will have ruptured and separated into three major layers of lipid, cytoplasm, and yolk going from top to bottom, with other minor layers at the interfaces (Fig. 1E). Cytoplasmic extract (see bracket in Fig. 1E) is collected using a syringe fitted with a 16-gauge beveled needle by puncturing the side of the tube at the bottom of the middle layer (see arrow in Fig. 1E) and very slowly drawing the cytoplasm while avoiding the interfaces. Carefully rotating the needle while puncturing the tube can facilitate perforation and avoid clogging the needle with plastic bits, but because the cytoplasm is turbid, it is important to note the beveled side of the needle to orient it upwards before starting to draw the cytoplasm. We typically recover ~20% of the original volume of dejellied eggs as cytoplasmic extract. Protease inhibitors are added to a final concentration of 10 μg/ml (each), energy mix for CSF extracts (20× CSF-energy mix) is added to a final concentration of 1×, and the extract is mixed gently by flicking or inverting.

Contamination of the cytoplasmic extract with material drawn from the interfaces and adjacent layers is not uncommon, and if we suspect this to be the case, we typically do a second spin as follows. The extract

is transferred to new centrifuge tubes of the same kind and appropriate size and spun again at 25,000*g* for 20 min at 4°. After this spin, the top and bottom layers formed by contaminating material will probably be small, but cytoplasm from the middle layer is still recovered by side puncture as described before. The resulting extract can be used immediately for assays requiring membranes, it can be used to prepare high speed supernatant, or it can be supplemented with sucrose to a final concentration of 200 m*M*, snap-frozen in liquid nitrogen and stored at −80°. For a five-frog prep, making cytoplasmic extract should take no longer than 2 h.

Making High-Speed Supernatant

Crude cytoplasmic extracts are very viscous and, therefore, must be diluted for efficient sedimentation during high-speed ultracentrifugation. The high-speed supernatant (HSS) must then be concentrated back to the original volume for use in actin assembly assays. The crude extract is diluted 10-fold in CSF-XB plus 10 μg/ml (each) PIs and 1 m*M* DTT. The dilute extract is then transferred to prechilled ultracentrifuge bottles (Beckman 355618), 0.5 ml of mineral oil (Sigma M-5904) is layered on top, and the extract is centrifuged at 500,000*g* for 1 h at 4° in a fixed-angle rotor (Beckman Type 70 Ti). Light membranes that do not pellet during high-speed centrifugation will partition into the mineral oil layer and are removed after centrifugation by aspirating the top layer carefully (the rotor and tubes should be handled with care to avoid mixing). A small amount of contamination with light membranes is unavoidable and does not adversely affect the quality of the HSS. The HSS is carefully recovered by pipetting without disturbing the pellet and filtered through 0.22-μm syringe filter units (Millipore SLGV 033) to avoid clogging the concentrator's filter during the subsequent step. The HSS is reconcentrated to the original cytoplasmic extract volume using Centriprep YM-10 Centrifugal Filter Units (Millipore 4304) according to the manufacturer's instructions. When more than one centrifugation step is required to achieve the desired volume, the retentate should be mixed between spins to avoid high local protein concentration. The HSS is recovered from the concentrator, centrifuged at 20,000*g* for 10 min at 4° to remove particulates, and 20× CSF-energy mix is added to a final concentration of 1×. The final protein concentration in the HSS should be ~25 mg/ml. The HSS can be used immediately for actin polymerization assays or supplement with sucrose to a final concentration of 200 m*M*, snap-frozen in 100- to 500-μl aliquots in liquid nitrogen, and stored at −80°. Making and concentrating HSS can take several hours.

Preparation of Prenylated Cdc42

Bacterially expressed Cdc42, which lacks the geranylgeranyl modification, fails to induce actin assembly in *Xenopus* egg HSS. We, therefore, use isoprenylated, tagged Cdc42 purified from the membranes of baculovirus infected *Spodoptera frugiperda* Sf9 cells by glutathione affinity chromatography. A protocol for purification of baculovirus-expressed untagged Cdc42 has been described in a previous volume of *Methods in Enzymology* Cerione *et al*., 1995), but because the protocol we routinely use is significantly different, we will describe it in detail here. For smaller scale purifications, the same basic procedure can also be done starting with transient transfections of a GST-Cdc42 construct into a number of different cell lines (293T, 293F, COS).

Reagents

Phosphate-buffered saline (PBS): 10 m*M* phosphate, pH 7.4, at 25°, 137 m*M* NaCl, 2.7 m*M* KCl.

Sonication buffer: 100 m*M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* Na-HEPES, pH 7.4, at 4°, 1 m*M* EDTA, 1 m*M* DTT, 0.1 m*M* GTP, 1 m*M* Phenylmethylsulfonyl Fluoride (PMSF, MP Biomedicals 800263, prepared as a 1 *M* stock in DMSO), 1× Complete EDTA-free protease inhibitor cocktail tablets (Roche Molecular Biochemicals 1873580), 10 μg/ml each leupeptin, pepstatin, and chymostatin.

Extraction buffer: 100 m*M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* Na-HEPES, pH 7.8, at 4°, 1 m*M* EDTA, 1 m*M* DTT, 0.1 m*M* GTP, 1 % Cholate, 1 m*M* PMSF, 10 μg/ml each leupeptin, pepstatin, and chymostatin.

Wash buffer 1: 100 m*M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* Na-HEPES, pH 7.8, at 4°, 0.1 m*M* EDTA, 1 m*M* DTT, 1% cholate.

Wash buffer 2: same as wash buffer 1 but 0.1 % cholate.

Loading buffer: 100 m*M* NaCl, 50 m*M* Na-HEPES, pH 7.8, at 4°, 20 m*M* EDTA, 1 m*M* DTT, 0.1% cholate.

Storage buffer: 100 m*M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* Na-HEPES, pH 7.8, at 4°, 0.1 m*M* EDTA, 10% glycerol, 1 m*M* DTT, 0.1% cholate.

Elution buffer: Storage buffer + 10 m*M* reduced glutathione, added from a 0.5 *M* stock of pH 8.0.

Procedure

Three liters of Sf9 cells are infected during log phase growth (density ~1 million cells/ml) with recombinant baculovirus bearing the sequence for human Cdc42 N-terminally fused to glutathione S-transferase (GST). Cells are harvested 72 h after infection by centrifugation and washed once with

phosphate-buffered saline (PBS). The cell pellet (~30 ml) is resuspended thoroughly in 5 volumes of cold sonication buffer. All subsequent steps are carried out at 4° or on ice unless indicated otherwise. Cells are lysed by sonication (7 × 10-sec pulses on output control setting 5 and duty cycle setting 50% for an analog Branson Sonifier 450 with a 19 mm diameter solid horn). An additional 1 m*M* PMSF is added to the lysate.

The lysate is centrifuged at 2000*g* for 20 min in conical tubes using a swinging bucket rotor. This low-speed spin separates nuclei and unbroken cells in the pellet (P1) from cytoplasm and membranes in the supernatant (S1). The pellet (P1) is very loose, so the supernatant (S1) should be recovered carefully by pipetting as opposed to decanting. For increased yield, the pellet (P1) can be resuspended in two volumes of sonication buffer, homogenized in a glass/glass homogenizer (Kontes Dounce Tissue Grinder 40 ml) using the large clearance pestle (pestle A), centrifuged as before, and the supernatant pooled with the rest of the supernatant (S1).

The supernatant (S1) is centrifuged at 300,000*g* for 1 h in a fixed-angle rotor (Beckman Type 70 Ti). This high-speed spin separates membranes containing prenylated Cdc42 in the pellet (P2) from cytosol in the supernatant (S2). Prenylated Cdc42 is extracted from the membranes as follows. The pellet (P2) is resuspended in 40 ml of extraction buffer and homogenized in a glass/glass homogenizer using the small clearance pestle (pestle B). The homogenate is transferred to a 50-ml conical tube, and extraction is allowed to proceed for 90 min with gentle rotation. The homogenate is then centrifuged at 400,000*g* for 35 min in a fixed-angle rotor. This clarifying spin separates membranes in the pellet (P3) from extracted prenylated Cdc42 in the supernatant (S3).

The supernatant (S3) is transferred to a 50-ml conical tube and is incubated with 1 ml of glutathione sepharose 4B beads (Amersham Biosciences 17-0756-01, pre-equilibrated in extraction buffer) for 3 h with gentle rotation. The beads are transferred to a 20-ml column (Biorad Econo-Pac 732-1010) and washed by gravity flow with 25 ml of wash buffer 1 followed by 25 ml of wash buffer 2.

We prefer to load prenylated Cdc42 with GDP or GTPγS while the protein is still bound to the beads, because this strategy provides an easy way to remove all unbound nucleotide, which can affect actin assembly assays if added to *Xenopus* egg extracts. The beads are washed by gravity flow with 25 ml of loading buffer, they are resuspended in 3 ml of loading buffer to make 4 ml of a 25% slurry, and half of the slurry is transferred to each of two 2-ml columns (Biorad Bio-Spin 732-6008) capped at the bottom. GDP or GTPγS (Roche Molecular Biochemicals) is added to a final concentration of 1.2 m*M*, the columns are capped tightly at both ends and incubated at 30° for 15 min, mixing by inversion every 2 min. $MgCl_2$ is

added to a final concentration of 30 m*M*, and the columns are incubated at 4° for 15 min mixing as before. The beads are drained and washed by gravity flow in the same column with 5 ml of storage buffer. A 50% slurry of the beads in storage buffer can be used directly in the microscopic rhodamine actin assay.

To elute bound Cdc42, the beads are drained and resuspended in the same column with 1.5 ml of elution buffer. The columns are capped tightly, and the elution reaction is allowed to proceed for 4 h at 4° with gentle rotation. The eluate is subsequently collected by gravity flow, and a second elution is carried out as before with another 1.5 ml of elution buffer. If the concentration of protein in the first elution is low (<0.1 mg/ml), the second elution can be done under harsher conditions by adjusting the pH of the elution buffer to 8.0, incubating at 4° for 1.5 h, heating to 30° for 10 min, and collecting the eluate immediately. The eluted protein is used directly in actin assembly assays, or it is snap frozen in aliquots and stored at −80°. Our typical yield is ~1 mg of eluted GST-Cdc42 protein, with a concentration range between 0.2–1 mg/ml. Because the GST tag does not affect the activity of Cdc42 in actin assembly assays, we generally do not cleave it. Furthermore, because the concentration of glutathione resulting after dilution of the eluted protein into the assays does not interfere with actin assembly, it is not necessary to dialyze it.

Preparation of Synthetic PI(4,5)P_2 Lipid Vesicles

Reagents

All lipids are purchased from Avanti Polar Lipids, Inc. and are stored at −80° in air-tight glass vials closed with Teflon-lined caps. (National Scientific Company B7800-1A).

Phosphatidylinositol-4,5-bisphosphate (PI[4,5]P_2): Cat. # 840046X, 5mg/ml in chloroform/methanol/water, MW = 1098.19.

Phosphatidylcholine (PC): Cat. #840051C, 25 mg/ml in chloroform, MW = 760.09.

Phosphatidylinositol (PI): Cat. # 840042C, 10 mg/ml in chloroform, MW =909.12.

Lipid buffer: 20 m*M* K-HEPES, pH 7.7, at 4°, 1 m*M* EDTA.

Procedure

To prepare 250 μl of PI(4,5)P_2-containing vesicles (PI[4,5]P_2:PC:PI, 4:48:48 molar ratio, 1 m*M* total lipid), solutions of PI(4,5)P_2 (2.2 μl), PC (3.65 μl) and PI (10.9 μl) are mixed in a glass test tube and dried under a stream of dry nitrogen. A thin film of lipid should be visible at the bottom of

the tube. The lipid is dried further under vacuum for 1 h to remove residual solvent. The dried lipid mixture is resuspended thoroughly in 250 μl of lipid buffer and then extruded 15 passes through a 0.1 μm pore polycarbonate membrane using the Mini-Extruder (Avanti Polar Lipids, Inc.). The resuspended lipid mixture should appear cloudy before extrusion and clear thereafter. Lipid vesicles are stored in the dark at 4° for up to 1 month.

Actin Polymerization Assays

We use two different assays to monitor Cdc42 and PI(4,5)P_2-induced actin polymerization in *Xenopus* egg extracts. In the first assay, we supplement the extracts with pyrene-labeled actin and follow the reaction kinetics in a fluorescence spectrophotometer. The fluorescence intensity of pyrene actin increases ~25-fold on polymerization (Kouyama and Mihashi, 1981), making this assay extremely useful for quantitative analysis of the kinetics of actin assembly (Fig. 2A). We have also adapted this assay for high-throughput screening of small molecule inhibitors of PI(4,5)P_2-mediated actin polymerization (Peterson *et al.*, 2001). In the second assay, we supplement the extracts with rhodamine-labeled actin and monitor the reaction visually under a fluorescence microscope. Unlike pyrene actin, the

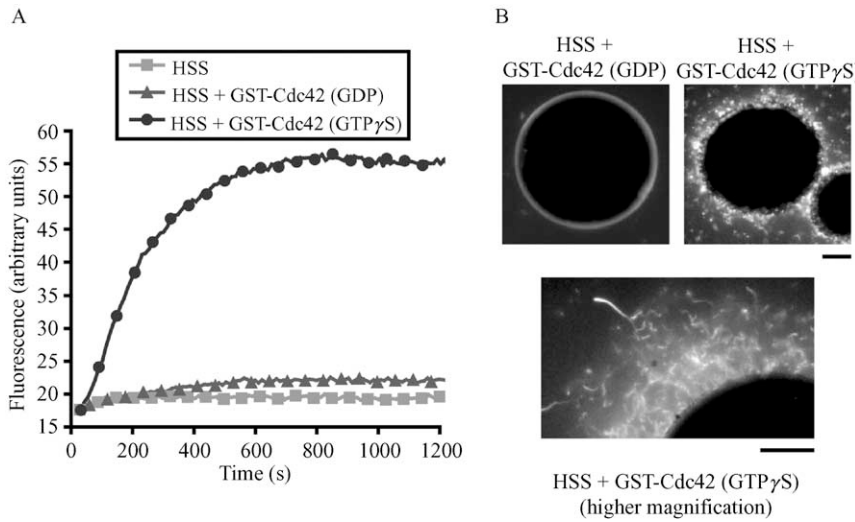

FIG. 2. Kinetic analysis of actin assembly in extracts supplemented with pyrene actin and microscopic analysis of actin assembly in extracts supplemented with rhodamine actin. (A) Prenylated GST-Cdc42 (200 n*M*) charged with GTPγS, but not GDP, induces actin polymerization. (B) Prenylated GST-Cdc42 on glutathione sepharose beads induces formation of actin foci and comet tails in a nucleotide-dependent manner. Scale bar = 25 μm.

fluorescence intensity of rhodamine actin does not increase on polymerization, and instead the observed variation in fluorescent signal is purely due to changes in local rhodamine actin concentration. However, in *Xenopus* egg extracts, Cdc42-induced actin polymerization results in the formation of large actin clusters shaped like foci and comet tails. These clusters are readily observable on the surface of GST-Cdc42 coated glutathione sepharose beads (Fig. 2B) or diffusing freely in reactions supplemented with soluble GST-Cdc42. Because the rhodamine actin assay is quick and requires only a few microliters of material, it is the assay of choice to follow actin polymerization activity during biochemical fractionation experiments.

Reagents

Pyrene actin: Prepared according to an established protocol (Kouyama and Mihashi, 1981) using rabbit skeletal muscle actin and *N*-(1-pyrene) iodoacetamide (Molecular Probes, Inc., P-29). A detailed protocol for preparing and characterizing pyrene actin has been described in an earlier volume of *Methods in Enzymology* (Zigmond, 2000). Pyrene actin can also be purchased from Cytoskeleton, Inc.

Rhodamine actin: Prepared according to an established protocol (Symons and Mitchison, 1991) using purified rabbit skeletal muscle actin and tetramethylrhodamine-5-iodoacetamide (Molecular Probes, Inc., T-6006). Rhodamine actin is also commercially available from Cytoskeleton, Inc.

20× CSF-energy mix: 150 m*M* creatine phosphate, 20 m*M* ATP, pH 7.0, 20 m*M* $MgCl_2$.

10× Assay buffer: 200 m*M* Hepes, pH 7.7, at 20°, 1 *M* KCl, 10 m*M* $MgCl_2$, 1 m*M* EDTA, 10 m*M* DTT.

Kinetic Analysis of Actin Assembly Using Pyrene Actin

A typical actin polymerization reaction is assembled as follows:

Volume added	Final concentration
20 μl *Xenopus* egg HSS (25 mg/ml total protein)	1:3 dilution of HSS
5 μl pyrene actin (1 mg/ml = 24 μM)	2 μM
3 μl 20× CSF-energy mix	1×
4 μl 10× assay buffer	1× (HSS is already in a buffer of similar composition)
+	
26 μl ddH_2O	
2 μl GST-Cdc42 (0.3 mg/ml eluted protein = 6 μM)	200 n*M*
or	
27.4 μl ddH_2O	
0.6 μl $PI(4,5)P_2$-containing vesicles (1 m*M* total lipid)	10 μM (total lipid)
60 μl total	

All components except for Cdc42 or PI(4,5)P_2-containing vesicles are added in the order listed, adjusting the indicated volumes based on actual protein concentrations. The reaction is mixed and incubated for 5 min at room temperature to allow the basal steady-state level of filamentous actin to stabilize (dilution of the extract and addition of exogenous pyrene actin can cause the F-actin basal level to shift). Cdc42 or PI(4,5)P_2 is added to initiate actin polymerization, the reaction is mixed, transferred immediately to a quartz cuvette (Cary Sub-Micro Fluorometer Cell, 40 μl, Varian 66-100216-00) and up to four reactions are monitored simultaneously in a fluorescence spectrophotometer (Varian Cary Eclipse) equipped with a Peltier Multicell Holder, using the provided kinetics software with the temperature control set to 20°. Pyrene fluorescence is measured at 407 nm with excitation at 365 nm.

Some dilution of the extract is necessary to reduce the level of autofluorescence, but diluting the extract too much may result in spontaneous polymerization. A 1:3 dilution of concentrated HSS generally works well, but because there is variability between different batches of extract, the optimal dilution must be determined empirically. The rate and extent of Cdc42 or PI(4,5)P_2-mediated actin polymerization also varies according to the quality of the extract, so the optimal concentration of agonist should be established empirically. A control reaction using GST-Cdc42 loaded with GDP should be conducted in parallel to account for nonspecific effects on actin polymerization. In this sample reaction, pyrene actin is added to a final concentration of 2 μM, but depending on the extent of pyrene labeling, between 1 and 3 μM can be added to obtain a good fluorescence signal. Because of the variability inherent in the preparation of reagents, reactions should only be compared when conducted the same day using the same batch of HSS, pyrene actin, and agonist. A representative assay for Cdc42-induced actin assembly is shown in Fig. 2A.

Microscopic Analysis of Actin Assembly Using Rhodamine Actin

A typical actin polymerization reaction is assembled as follows:

4 ul *Xenopus* egg HSS (25 mg/μl total protein)
0.5 ul rhodamine actin (1 mg/μl)
0.35 μl 20× CSF-energy mix
1.65 μl 1× assay buffer
0.5 μl GST-Cdc42 (50% slurry of glutathione sepharose beads or 0.3 mg/ml eluted protein)

7 μl total

All components except for Cdc42 are added in the order listed, mixed, and incubated for 5 min at room temperature to allow the basal steady-state level of F-actin to stabilize. Cdc42 is added to initiate polymerization, and 5 μl of the reaction mixture are squashed between a slide and a coverslip. The sample is immediately mounted on the stage of a fluorescence microscope and visualized or imaged using an appropriate filter set for rhodamine. Depending on the quality of the HSS, many foci and occasional comet tails should begin to appear 15 sec to 2 min after addition of GTPγS loaded GST-Cdc42. Although in our experience the formation of actin foci in response to Cdc42 is highly reproducible, background rhodamine fluorescence can vary depending on a number of factors, including the quality of the extract and proteins used. Control experiments to test the nucleotide dependence of the response to GST-Cdc42 and to test the cytochalasin D sensitivity of the fluorescent clusters should, therefore, be performed in parallel. Representative images of an assay in which actin assembly was induced by GST-Cdc42 on glutathione sepharose beads are shown in Fig. 2B.

Functional Analysis of Actin Assembly by Immunodepletion and Rescue

Immunodepletion is a powerful technique that allows the functional importance of a molecule of interest to be assessed in *Xenopus* egg extracts. The specificity of immunodepletion can be confirmed by adding back recombinant protein to ''rescue'' the biological activity under investigation. Furthermore, structure–function analysis can be conducted by testing the activities of recombinant proteins added back to the immunodepleted extracts. During the course of our study of the Cdc42-actin signaling pathway, we used immunodepletion to demonstrate the functional requirement of both N-WASP and Toca-1 for Cdc42-mediated actin nucleation in *Xenopus* egg extracts (Rohatgi *et al*., 1999; Ho *et al*., 2004) (see Fig. 3 for Toca-1 immunodepletion experiments).

The quality of the antibody used for depletion is the single most important factor determining the ability to deplete a specific protein or protein complex from the extract. In our experience, antibodies that work best for immunodepletion are prepared by immunizing rabbits with recombinant full-length proteins. We commonly use antibodies that have been affinity purified. Crude serum can be used directly if the titer of the specific antibody is sufficiently high. The use of crude serum is preferable in some cases, given that polyclonal antibodies with the highest affinity are sometimes poorly recovered after affinity purification.

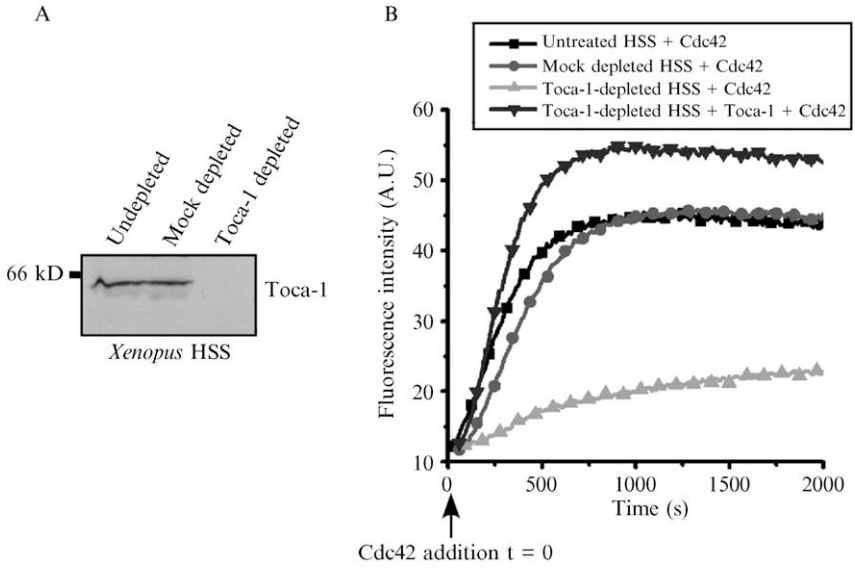

FIG. 3. Immunodepletion of Toca-1 from *Xenopus* egg extract. (A) Western blot analysis showing depletion of Toca-1 from *Xenopus* egg HSS. (B) Pyrene actin assay demonstrating the functional requirement of Toca-1 for Cdc42-mediated actin nucleation in *Xenopus* egg extracts. Addition of recombinant Toca-1 to the Toca-1 depleted extract "rescues" the ability of Cdc42 to induce actin polymerization. (Reprinted with permission from Ho *et al.*, 2004.

Immunodepletion of Xenopus *Egg Extracts*

Seven micrograms of antibody, 25 μl of protein A–coupled magnetic Dynabeads (Dynal Biotech 100.01), and 100 μl of PBS containing 0.1% Triton X-100 are mixed in a 0.5-ml Eppendorf tube by gentle rotation for 1 h at room temperature. We use a magnetic particle concentrator (Dynal Biotech MPC) to retrieve the beads during the following steps. The beads are washed twice with 100 μl of PBS containing 0.1% Triton X-100 and three times with 100 μl of 1$\times$ assay buffer. These washes are necessary to remove all unbound antibody and detergent, which can affect subsequent assays using the extracts. Because the beads tend to stick to the tube in the absence of detergent, one should proceed promptly after the washes. After the final wash, all buffer is removed, and 100 μl of *Xenopus* egg HSS is added to the beads. The mixture is incubated for 90 min at 4°, mixing every 20 min. Depending on the antibody, it is sometimes necessary to perform a second round of immunodepletion using fresh antibody-coupled beads.

After the beads are retrieved using the magnetic particle concentrator, the HSS is transferred to a clean tube and centrifuged briefly to remove any residual beads. The supernatant is transferred once more and stored on ice until use.

For actin assembly assays, we use the depleted extracts within 6 h after immunodepletion. Because the immunodepletion procedure can affect the extract, a mock depletion using preimmune IgG or random IgG from the same species should be performed in parallel and taken into consideration when interpreting immunodepletion results. Depletion of the protein of interest can be assessed by Western blot (Fig. 3A). An experiment in which endogenous Toca-1 was immunodepleted from *Xenopus* egg HSS and recombinant Toca-1 protein was added back to rescue Cdc42-mediated actin assembly is shown in Fig. 3B.

Concluding Remarks

In this chapter, we have described a set of tools for studying signal-dependent actin assembly in a biochemically amenable physiological system. We hope we have conveyed how *Xenopus* egg extracts can serve as a powerful system to study regulated actin assembly, both as an engine for the discovery of novel pathway components and as a versatile biochemical tool to investigate pathway mechanisms. Reconstitution of other signal transduction pathways in these extracts should allow detailed biochemical investigations of the kind we have conducted on the $PI(4,5)P_2$ and Cdc42 pathway.

Acknowledgments

We thank Andrew Horwitz, Jeffrey Peterson, Rajat Rohatgi, and Orion Weiner for comments on the manuscript. The studies described in this chapter were supported by grants from The National Institutes of Health to M. W. K.

References

Cerione, R. A., Leonard, D., and Zheng, Y. (1995). Purification of baculovirus-expressed Cdc42Hs. *Methods Enzymol.* **256,** 11–15.

Ho, H. Y., Rohatgi, R., Lebensohn, A. M., Ma, L., Li, J., Gygi, S. P., and Kirschner, M. W. (2004). Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. *Cell* **118,** 203–216.

Hutchison, C., and Kill, I. (1989). Changes in the nuclear distribution of DNA polymerase alpha and PCNA/cyclin during the progress of the cell cycle, in a cell-free extract of Xenopus eggs. *J. Cell Sci.* **93**(Pt 4), 605–613.

Kouyama, T., and Mihashi, K. (1981). Fluorimetry study of N-(1-pyrenyl)iodoacetamide-labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. *Eur. J. Biochem.* **114,** 33–38.

Ma, L., Cantley, L. C., Janmey, P. A., and Kirschner, M. W. (1998a). Corequirement of specific phosphoinositides and small GTP-binding protein Cdc42 in inducing actin assembly in *Xenopus* egg extracts. *J. Cell Biol.* **140,** 1125–1136.

Ma, L., Rohatgi, R., and Kirschner, M. W. (1998b). The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. *Proc. Natl. Acad. Sci. USA* **95,** 15362–15367.

Marchand, J. B., Moreau, P., Paoletti, A., Cossart, P., Carlier, M. F., and Pantaloni, D. (1995). Actin-based movement of Listeria monocytogenes: Actin assembly results from the local maintenance of uncapped filament barbed ends at the bacterium surface. *J. Cell Biol.* **130,** 331–343.

Murray, A. W. (1991). Cell cycle extracts. *Methods Cell Biol.* **36,** 581–605.

Murray, A. W., and Kirschner, M. W. (1989). Cyclin synthesis drives the early embryonic cell cycle. *Nature* **339,** 275–280.

Newport, J., and Spann, T. (1987). Disassembly of the nucleus in mitotic extracts: Membrane vesicularization, lamin disassembly, and chromosome condensation are independent processes. *Cell* **48,** 219–230.

Ohsumi, K., Katagiri, C., and Kishimoto, T. (1993). Chromosome condensation in *Xenopus* mitotic extracts without histone H1. *Science* **262,** 2033–2035.

Peterson, J. R., Lokey, R. S., Mitchison, T. J., and Kirschner, M. W. (2001). A chemical inhibitor of N-WASP reveals a new mechanism for targeting protein interactions. *Proc. Natl. Acad. Sci. USA* **98,** 10624–10629.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M. W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97,** 221–231.

Sawin, K. E., Le Guellec, K., Philippe, M., and Mitchison, T. J. (1992). Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature* **359,** 540–543.

Symons, M. H., and Mitchison, T. J. (1991). Control of actin polymerization in live and permeabilized fibroblasts. *J. Cell Biol.* **114,** 503–513.

Theriot, J. A., Rosenblatt, J., Portnoy, D. A., Goldschmidt-Clermont, P. J., and Mitchison, T. J. (1994). Involvement of profilin in the actin-based motility of L. monocytogenes in cells and in cell-free extracts. *Cell* **76,** 505–517.

Zigmond, S. H. (2000). *In vitro* actin polymerization using polymorphonuclear leukocyte extracts. *Methods Enzymol.* **325,** 237–254.