Phosphorylation of the Arp2/3 complex is necessary to nucleate actin filaments

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The actin-related protein 2/3 (Arp2/3) complex is the primary nucleator of new actin filaments in most crawling cells. Nucleation-promoting factors (NPFs) of the Wiskott-Aldrich syndrome protein (WASP)/Scar family are the currently recognized activators of the Arp2/3 complex. We now report that the Arp2/3 complex must be phosphorylated on either threonine or tyrosine residues to be activated by NPFs. Phosphorylation of the Arp2/3 complex is not necessary to bind NPFs or the sides of actin filaments but is critical for binding the pointed end of actin filaments and nucleating actin filaments. Mass spectrometry revealed phosphorylated Thr237 and Thr238 in Arp2, which are evolutionarily conserved residues. In cells, phosphorylation of only the Arp2 subunit increases in response to growth factors, and alanine substitutions of Arp2 T237 and T238 or Y202 inhibits membrane protrusion. These findings reveal an additional level of regulation of actin filament assembly independent of WASP proteins, and show that phosphorylation of the Arp2/3 complex provides a logical "or gate" capable integrating diverse upstream signals.

Introduction

Eukaryotic cells change shape and move by assembling ordered networks of actin filaments at their leading edges (Pollard et al., 2001). The heart of this actin-based motility engine is the actin-related protein 2/3 (Arp2/3) complex, a seven-subunit protein complex that builds cross-linked filament arrays by nucleating new filaments from the sides of preexisting filaments (Goley and Welch, 2006). Actin-nucleating and cross-linking activities of the Arp2/3 complex are required to build many cellular structures, including lamellipodia and phagocytic cups, and to move endosomes and intracellular pathogens through the cytoplasm (Pollard and Borisy, 2003). The Arp2/3 complex, which is composed of two actin-related proteins (Arp2 and Arp3) and five accessory proteins, nucleates a new actin filament by first binding to the side of a preexisting filament. Next, the Arp2 and Arp3 subunits form a protonucleus that mimics a new fast-growing barbed end of an actin filament (Robinson et al., 2001; Goley et al., 2004; Nolen et al., 2004). Actin monomers polymerize at this barbed end, elongating rapidly toward the plasma membrane, whereas the Arp2/3 complex remains bound to the slow-growing pointed end, thereby cross-linking the new filament to the mother filament (Mullins et al., 1998). Arp2 and Arp3 subunits of the complex bind ATP. Although ATP hydrolysis is not necessary for actin nucleation, it is thought to be necessary for debranching of Arp2/3-branched actin filaments (Martin et al., 2006). Wiskott-Aldrich syndrome protein (WASP) family proteins are the predominant activators of the Arp2/3 complex. However, in addition to regulation by WASP proteins, recent evidence indicates the Arp2/3 complex is phosphorylated by serine/threonine kinases. The p21-activated kinase (PAK) phosphorylates Thr21 in the ARPC1 subunit (Vadlamudi et al., 2004), and the MAPK-activated protein kinase 2 phosphorylates Ser77 in the ARPC5 subunit (Singh et al., 2003). Although phosphorylation of the Arp2/3 complex is suggested to increase cell motility, whether phosphorylation directly regulates actin nucleating activity of the Arp2/3 complex has not been reported.

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Abbreviations used in this paper: AP, Antarctic phosphatase; Arp, actin-related protein; NPF, nucleation-promoting factor; PP2Cα, protein phosphatase 2Cα; pThr, phosphothreonine; pTyr, phosphotyrosine; WASP, Wiskott-Aldrich syndrome protein.

The online version of this article contains supplemental material.

Results and discussion

Purified Arp2/3 complex is phosphorylated

We found that several subunits of the Arp2/3 complex are phosphorylated in cells. Using 2D electrophoresis of purified

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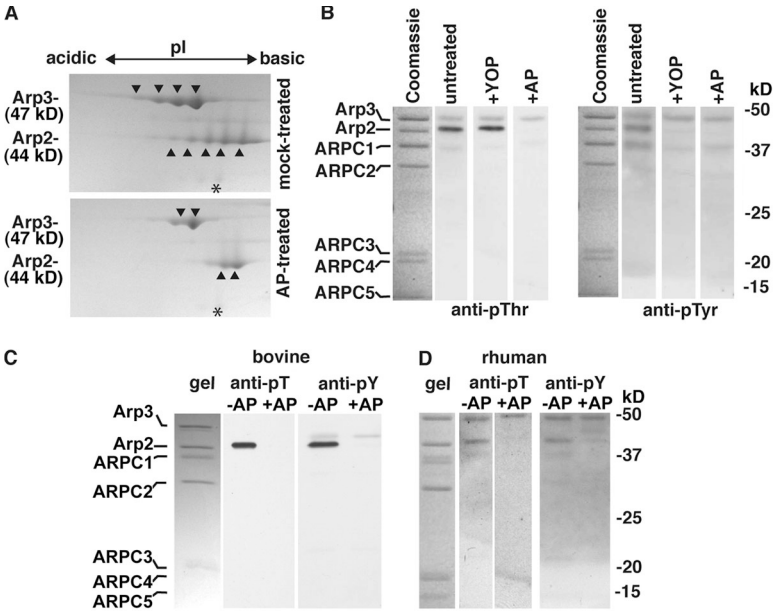

Figure 1. **The Arp2/3 complex is threonine- and tyrosine-phosphorylated in cells.** (A) The Arp2/3 complex purified from *A. castellani* separated by 2D electrophoresis revealed that Arp2 migrated as five spots and Arp3 as four spots (arrowheads). Pretreatment of the complex with the dual-specificity AP resulted in Arp2 migrating as two spots and Arp3 migrating as two spots at a more basic pI relative to a fiduciary marker (asterisks). (B) The Arp2, Arp3, and ARPC1 subunits of the Arp2/3 complex purified from *A. castellani* labeled with antibodies to pThr and pTyr. Dephosphorylation of the Arp2/3 complex with AP abolished labeling of Arp2 with pThr and pTyr antibodies, decreased labeling of ARPC1 by pTyr antibodies to ARPC1, and did not change labeling of Arp3. Dephosphorylation with the tyrosine-specific phosphatase YOP did not affect labeling of pThr antibodies but abolished labeling of Arp2 with pTyr antibodies and decreased labeling of ARPC1. (C) The Arp2 subunit of purified bovine Arp2/3 complex labeled with antibodies to both pThr and pTyr. (D) The Arp3 and Arp2 subunits of recombinant human Arp2/3 complex labeled with antibodies to pThr and pTyr.

Arp2/3 complex from *Acanthamoeba castellani*, Arp2 and Arp3 migrated as multiple spots (Fig. 1 A). After dephosphorylation with Antarctic phosphatase (AP), a dual-specificity alkaline phosphatase, Arp2 and Arp3 migrated as fewer spots at a more basic pI. These data suggest multiple phosphorylated species of Arp2 and at least one of Arp3. The presence of multiple spots for both subunits after AP treatment suggests posttranslational modifications in addition to phosphorylation.

We also used immunoblotting to determine phosphorylation of the Arp2/3 complex purified from *A. castellani*. Arp2, Arp3, and ARPC1 subunits labeled with antibodies to phosphothreonine (pThr) and phosphotyrosine (pTyr; Fig. 1 B). Labeling of ARPC1 with pThr antibodies is consistent with a previous report that this subunit is phosphorylated on Thr21 (Vadlamudi et al., 2004). When the *A. castellani* complex was dephosphorylated with AP, Arp2 labeling with pThr and pTyr antibodies was abolished, ARPC1 labeling with pTyr antibodies was decreased, and Arp3 labeling was unchanged. Because AP changed the mobility of Arp3 on 2D gels but not Arp3 immunolabeling, we suspect the immunoblot signal may be nonspecific. Arp3 could be phosphorylated on serine residues; however, we were unable to confirm specific labeling of any Arp2/3 complex subunit with antiphosphoserine antibodies. Tyrosine phosphorylation of Arp2 and ARPC1 subunits was confirmed by dephosphorylating the Arp2/3 complex with the tyrosine-specific phosphatase from *Yersinia entercolitica* (YOP; Fig. 1 B). Using Arp2/3 complex purified from bovine thymus, the Arp2 but not the Arp3 or ARPC1 subunits labeled with antibodies to pThr and pTyr, and labeling was abolished after treatment with AP (Fig. 1 C). Immunoblotting recombinant human Arp2/3 complex purified from insect cells also showed pThr and pTyr labeling of Arp2 before but not after AP treatment (Fig. 1 D). These data suggest that phosphorylation of the Arp2/3 complex, particularly Arp2, is not species specific.

Arp2/3 complex phosphorylation is required for actin nucleation

We found that a dephosphorylated Arp2/3 complex does not nucleate actin filaments. Mock-treated (heat-inactivated phosphatase) Arp2/3 complex purified from *A. castellani* nucleated actin filaments with the C-terminal domain of the nucleation-promoting factor (NPF) Scar (ScarVCA) faster than with actin alone (Fig. 2 A), and at rates similar to an untreated complex (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200802145/DC1). We calculated the number of barbed ends formed by 5 nM of mock-treated Arp2/3 complex, 500 nM ScarVCA, and 4 μM actin to be ~3 nM. The number of free barbed ends is comparable to similar studies (Higgs et al., 1999) and in agreement with ScarVCA being a less potent Arp2/3 activator compared with N-WASP-VCA (Zalevsky et al., 2001). The Arp2/3 complex dephosphorylated with AP did not nucleate actin filaments with actin alone or in the presence of ScarVCA (Fig. 2 A) or N-WASP VCA (Fig. S1 B). Arp2/3 nucleating activity decreased with increasing time of incubation with the phosphatase (Fig. S1 C). Activity of the Arp2/3 complex incubated with AP in the presence of phosphatase inhibitors was identical to the activity of mock-treated controls (Fig. S1 D). Arp2/3 complex purified from bovine thymus also nucleated actin filaments but not after treatment with AP (Fig. S1 E).

We next asked whether tyrosine or serine/threonine phosphorylation is necessary for Arp2/3 nucleation activity. Arp2/3 complex purified from *A. castellani* and dephosphorylated with YOP or the serine/threonine protein phosphatase 2Cα (PP2Cα) nucleated actin at rates identical to mock-treated controls (Fig. 2, B and C). However, when samples were dephosphorylated in series with YOP and PP2Cα, the Arp2/3 complex was unable to nucleate actin filaments (Fig. 2 D), which suggests that either tyrosine phosphorylation or serine/threonine phosphorylation is required. These findings indicate that regulation of actin nucleation by the Arp2/3 complex may act as a logical "or gate," with

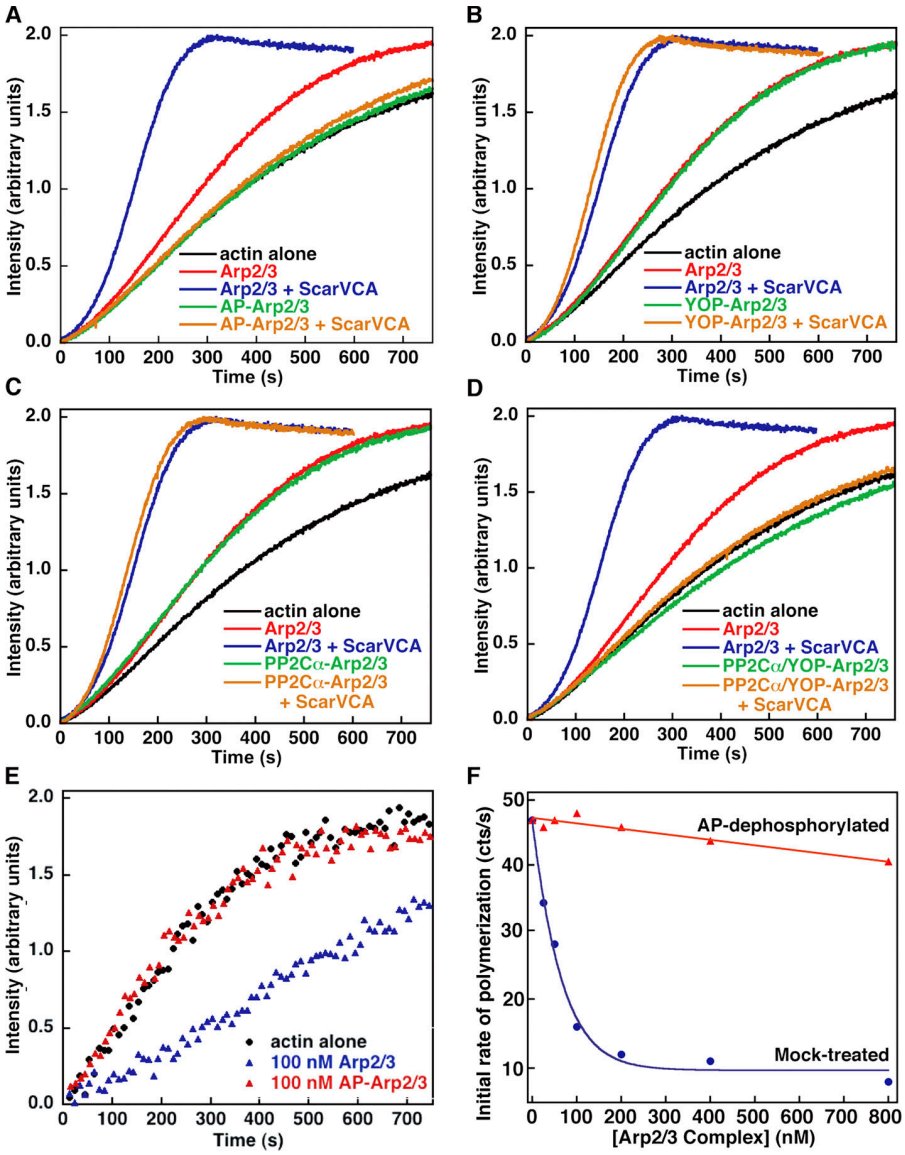

Figure 2. **The Arp2/3 complex must be phosphorylated to nucleate actin filaments.** (A) Polymerization assays show that Arp2/3 complex in the absence (red) and presence of 500 nM ScarVCA (blue) nucleates actin faster than actin alone (black). Arp2/3 complex dephosphorylated with the dual-specificity phosphatase AP (AP-Arp2/3) did not nucleate actin filaments in the absence (green) or presence of ScarVCA (orange). (B and C) Arp2/3 complex dephosphorylated with the tyrosine-specific phosphatase YOP (YOP-Arp2/3; B) or the serine/threonine phosphatase PP2Cα (PP2Cα-Arp2/3; C) nucleated actin filaments at rates similar to control Arp2/3 complex in the absence (green) or presence of ScarVCA (orange). (D) Arp2/3 complex dephosphorylated in series with YOP and PP2Cα (PP2Cα/YOP-Arp2/3) did not nucleate actin filaments either alone (green) or with ScarVCA (orange). (E) Phosphorylation of the Arp2/3 complex is necessary to cap the pointed ends of actin filaments. 100 nM actin filaments capped at the barbed end with gelsolin were capped at the pointed end by mock-treated Arp2/3 complex (blue circles) but not by AP-dephosphorylated Arp2/3 complex (red triangles). (F) Gelsolin-capped actin filaments were titrated with mock-treated Arp2/3 complex (blue) or AP-dephosphorylated Arp2/3 complex.

either tyrosine or serine/threonine phosphorylation being necessary for activation.

Phosphorylation of the Arp2/3 complex is necessary to cap the pointed ends of actin filaments

Phosphorylation could control nucleation activity by regulating several properties, including the association of subunits, the affinity of the Arp2/3 complex for NPFs, the affinity of the complex for the sides of actin filaments, and/or the ability of the complex to undergo the conformational change required for nucleation. Affinity purification using N-WASP VCA bound to agarose beads indicated an intact complex with and without AP treatment (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200802145/DC1). These data also indicated that AP-treated Arp2/3 complex binds to NPFs and we found that phosphorylation did not affect the affinity of the Arp2/3 complex for binding ScarVCA (Fig. S2 B) or N-WASP VCA (Fig. S2 C). The affinity of untreated and mock-treated Arp2/3 complex for ScarVCA (K_d = 0.88 μM) and for N-WASP VCA (K_d = 0.34 μM) was similar to previous findings (Pan et al., 2004; Kelly et al., 2006) and did not change with AP treatment. Additionally, phosphorylation did not affect affinity of the Arp2/3 complex for actin filaments, as determined by cosedimentation with filamentous actin. The Arp2/3 complex untreated or dephosphorylated with AP (Fig. S2 D), YOP, PP2Cα, or YOP and PP2Cα (unpublished data) bound actin filaments with a K_d of ~1 μM, which is consistent with published data (Mullins et al., 1998).

We used pointed-end binding to assay for the ability of the complex to undergo the conformational change required for nucleation. Nucleation of a new actin filament or binding of Arp2/3 complex to the pointed end of preformed actin filaments induces nucleotide hydrolysis on the Arp2 subunit (Dayel and Mullins, 2004). Actin filaments capped at the barbed end with gelsolin (100 nM gelsolin-actin seeds) elongated from the pointed end in the absence of Arp2/3 complex and excess actin monomer (Fig. 2 E). The addition of mock-treated Arp2/3 complex, a pointed end–capping protein (Mullins et al., 1998), slowed pointed-end elongation.

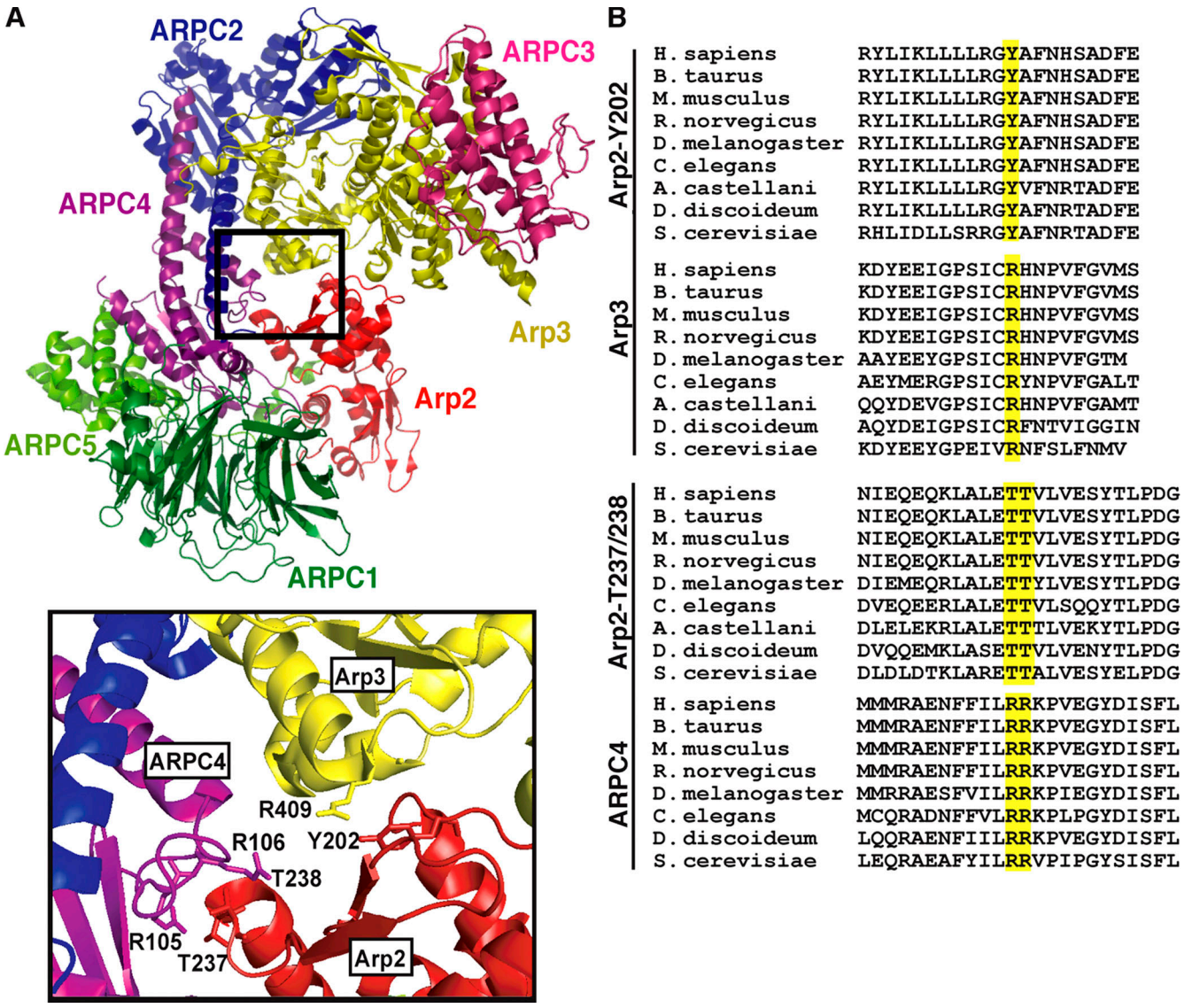

Figure 3. **The putative phosphorylation sites are conserved.** (A) Phosphorylated T237, T238, and Y202 of Arp2 were mapped to the Arp2/3 complex crystal structure obtained by Robinson et al. (2001). From the crystal structure, T237 and T238 of Arp2 are adjacent to R105 and R106 of ARPC4, and Y202 of Arp2 is adjacent to R409 of Arp3. (inset) Enlarged view of the boxed region. (B) The proposed phosphorylation sites on the Arp2 subunit and adjacent arginine residues in ARPC4 and Arp3 are conserved in nine organisms.

Mock-treated Arp2/3 complex bound pointed ends with an apparent K_d of ~42 nM, which is similar to previously described values (Mullins et al., 1998). The Arp2/3 complex dephosphorylated with AP did not block elongation of barbed end–capped actin filaments. The rate of pointed-end elongation decreased when titrated with untreated Arp2/3 complex but remained nearly constant when titrated with AP-dephosphorylated Arp2/3 complex (Fig. 2 F). Because capping of the actin filament pointed end is similar to the nucleation step, these data indicate that the complex must be phosphorylated to undergo the conformation change required for actin nucleation.

Phosphorylation of the Arp2 subunit is on conserved residues and is regulated in cells

We used nanoelectrospray LC-MS/MS to identify phosphorylated residues in Arp2/3 complex purified from *A. castellani* cells. We identified two threonine phosphorylation sites on the Arp2 subunit, T237 and T238 (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200802145/DC1), but did not detect pThr residues in Arp3 or pSer residues in Arp2 or Arp3. We also did not detect pTyr residues on Arp2 or Arp3, despite immunoblotting data indicating that Arp2 labels with anti-pTyr antibodies before but not after treatment with AP or YOP. Because the amino acid sequence of *A. castellani* ARPC1 is not known, we were unable to identify phosphorylated sites by mass spectrometry.

Based on the atomic structure of the Arp2/3 complex determined by Robinson et al. (2001), T237 and T238 of the Arp2 subunit are in subdomain IV near the interface of the Arp3, ARPC2, and ARPC4 subunits (Fig. 3 A). The side chains of Arp2 T237 and T238 are directly adjacent to the basic side chains of R105 and R106, respectively, in the ARPC4 subunit (Fig. 3 A). Phosphorylated T237 and T238 of the Arp2 complex could form salt

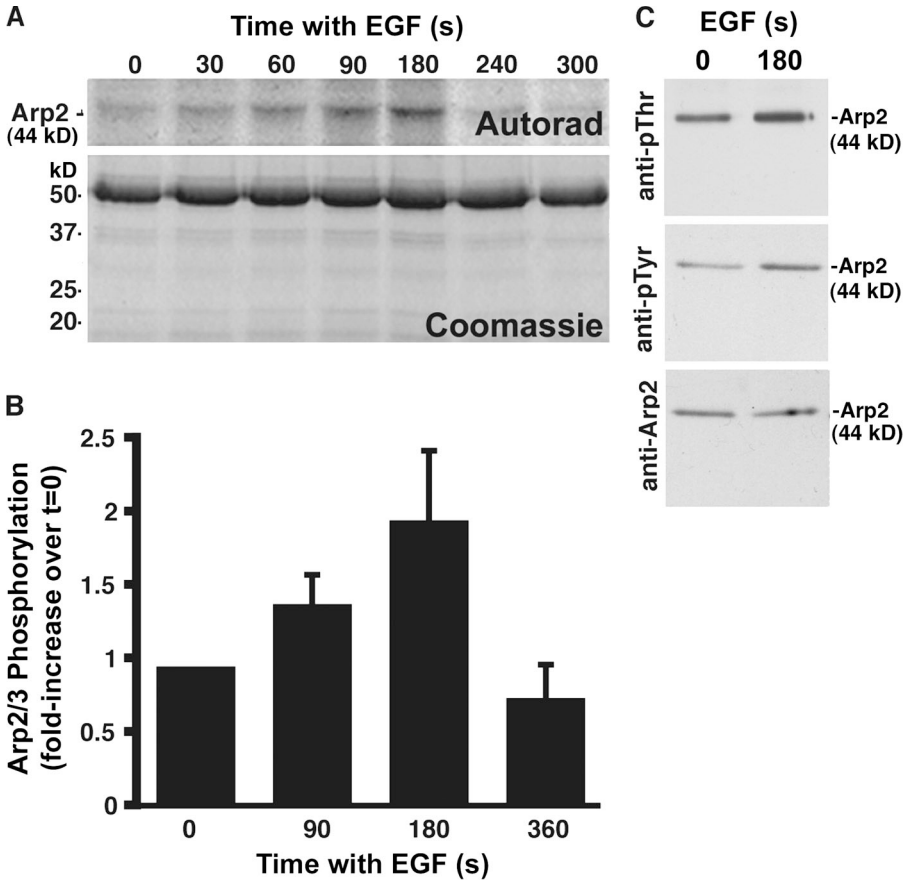

Figure 4. **Phosphorylation of the Arp2 is regulated in cells.** (A) Immunoprecipitation of the Arp2/3 complex with antibodies to ARPC1 from MTLn3 epithelial cells metabolically labeled with [^{32}P]orthophosphate indicated that the Arp2 subunit is phosphorylated in unstimulated cells, and phosphorylation increased after stimulation with EGF. (B) Quantification of time-dependent Arp2 phosphorylation. Data are expressed as the fold increase of phosphorylation in the absence of EGF and represent means ± SD of five separate cell preparations. (C) The Arp2/3 complex was immunoprecipitated from quiescent and EGF-stimulated MTLn3 cells. Labeling of Arp2 with antibodies to pThr and pTyr increased ~1.3- and 1.21-fold after EGF stimulation, respectively.

bridges with R105 and R106 of ARPC4 to perhaps stabilize the Arp2/3 complex in an active conformation. Molecular dynamics simulations suggest that Arp2 pT237 interacts exclusively with R105 of ARPC4 (unpublished data). Because Arp2 pTyr residues were suggested by immunoblotting but were not identified by mass spectrometry, we searched the Arp2 structure for potential tyrosine phosphorylation sites that might interact with basic residues of adjacent subunits. Of particular interest was Y202 in subdomain IV, which is adjacent to R409 in the Arp3 subunit. Sequence alignments of Arp2/3 subunits of nine organisms revealed that Arp2 T237, T238, and Y202; Arp3 R409; and ARPC4 R105 and R106 are evolutionary conserved from yeasts and slime molds to mammals (Fig. 3 B).

To determine whether phosphorylation of Arp2/3 complex subunits is regulated in cells, MTLn3 epithelial carcinoma cells were metabolically labeled with [^{32}P]orthophosphate and stimulated with 10 nM EGF, and the Arp2/3 complex was immunoprecipitated with antibodies to the ARPC1 subunit. Phosphorylation of only the Arp2 subunit was detected in unstimulated and EGF-treated cells (Fig. 4 A). Phosphorylation of other unidentified proteins was detected in the immune complex; however, none of these proteins migrated with Arp2/3 complex subunits. EGF induced a time-dependent but transient increase in Arp2 phosphorylation (Fig. 4, A and B). Phosphorylation rapidly increased at 90 s, was maximal at 180 s, with a twofold increase, and was not significantly different compared with quiescence at 360 s. In response to EGF, labeling by anti-pThr increased 1.3-fold and labeling by anti-pTyr increased 1.21-fold at 180s, as determined by immunoprecipitating Arp2/3 complex from unlabeled cells and immunoblotting with antibodies to pThr and pTyr (Fig. 4 C).

Phosphorylation of Arp2 is necessary for lamellipodia formation

We asked whether the identified pThr residues and the speculated pTyr residue in Arp2 were functionally significant by analyzing lamellipodia formation by *Drosophila melanogaster* Schneider S2 cells. When plated on coverslips coated with concanavalin A, S2 cells extended a circular, flat lamellipodia (Fig. 5 A), which is similar to previous findings (Rogers et al., 2003). When the Arp2/3 complex is inactivated by depletion of the ARPC3 or ARPC4 subunits by RNAi, S2 cells exhibit a stellate morphology with formations that extend radially around the cell body (Rogers et al., 2002; Iwasa and Mullins, 2007). We observed a similar stellate phenotype when S2 cells were depleted of the Arp2 subunit by Arp2 siRNA (Fig. 5 A). Transient expression of wild-type Arp2 fused to GFP (Arp2-WT-GFP) in Arp2-depleted cells restored a wild-type phenotype with flat circular lamellipodia (Fig. 5, A and B). Expression of Arp2 containing alanine substitutions for T237 and T238 (Arp2-T237/238A-GFP) or for Y202 (Arp2-Y202A-GFP) also restored lamellipodia formation (Fig. 5, A and B). In contrast, expression of Arp2-T237/238A-Y202A-GFP did not restore lamellipodia formation, and cells retained a stellate phenotype. These findings are consistent with our in vitro actin polymerization data suggesting that either threonine or tyrosine phosphorylation of the complex is necessary for nucleating activity.

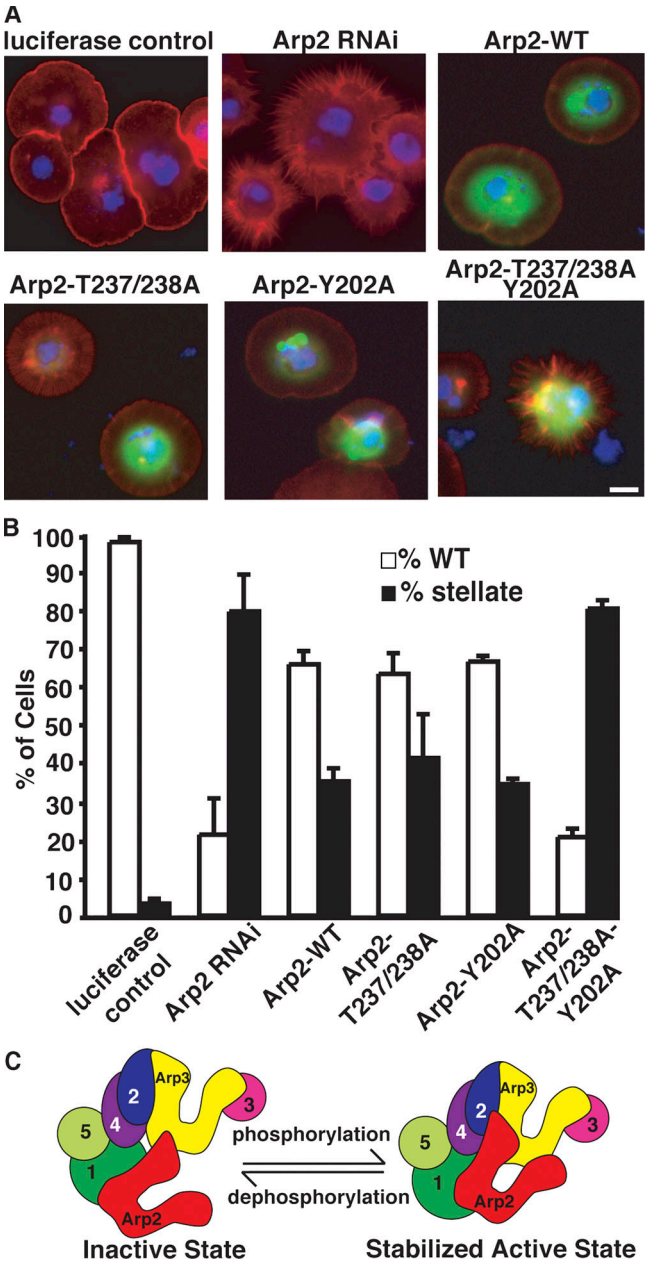

Figure 5. **Phosphorylation of Arp2 is necessary for lamellipodia formation.** (A) Control, luciferase RNAi-treated *D. melanogaster* S2 cells on concanavalin A–coated coverslips spread symmetrical, round lamellipodia. S2 cells depleted of the Arp2 subunit by siRNA (Arp2 RNAi) exhibited a stellate morphology with membrane protrusions radiating from the cell body. A wild-type morphology of circular lamellipodia was restored in Arp2-depleted S2 cells expressing wild-type Arp2-GFP (Arp2-WT-GFP), or mutated Arp2-T237/238A-GFP or Arp2-Y202A-GFP. Arp2-depleted S2 cells expressing the triple mutant Arp2-T237/238A-Y202A-GFP remained stellate. Bar, 5 µM. (B) The number of cells with wild-type or stellate morphology were scored for the indicated conditions and expressed as the mean ± SD of three separate cell preparations, with >500 cells scored for each condition per cell preparation. (C) Model for activation of the Arp2/3 complex by phosphorylation of the Arp2 subunit. In the "inactive" form of the Arp2/3 complex, the Arp2 and Arp3 subunits are splayed open. Phosphorylated T237/T238 and Y202 residues in Arp2 interact with arginine residues on adjacent subunits to maintain a conformation that stabilizes the complex in the "active" form. This conformation aligns the Arp2 and Arp3 subunits to form a protonucleus for actin nucleation.

We also confirmed that recombinant wild-type but not mutant Arp2 in S2 cells was phosphorylated on threonine and tyrosine residues (Fig. S3, B and C). Complexes immunoprecipitated with antibodies to GFP and immunoblotted with antibodies to pThr and pTyr showed labeling of Arp2-WT but not Arp2-T237/238-Y202A, which suggests that Y202 is necessary for tyrosine phosphorylation of Arp2 in cells. Whether this reflects a direct requirement for Y202 phosphorylation or a necessary role of Y202 for phosphorylation of a different Tyr residue in Arp2 remains to be determined.

Our data show that the Arp2/3 complex must be phosphorylated on threonine or tyrosine residues for actin-nucleating activity. Phosphorylation is necessary but not sufficient for nucleating activity of the complex, which must be stimulated by NPFs. However, NPFs are not sufficient in the absence of Arp2/3 complex phosphorylation. In cells, lamellipodia formation is inhibited by alanine substitutions in Arp2 T237/238-Y202. Whether phosphorylation of these residues is necessary for nucleating activity of the complex or whether they might function as docking sites for a kinase remains to be determined. However, based on their potential charge interactions with other subunits of the complex, we predict that phosphorylation of Arp2 T237/238 or Y202 stabilizes the Arp2/3 complex in an active conformation (Fig. 5 C). Although current models of Arp2/3 complex regulation do not incorporate a role for phosphorylation of subunits (Goley and Welch, 2006; Stradal and Scita, 2006), a requirement for either threonine or tyrosine phosphorylation is an effective mechanism for integrating diverse upstream signals regulating actin filament assembly.

Materials and methods

Protein purification

Arp2/3 complex and actin from *A. castellani* were purified as described previously (Zalevsky et al., 2001). Bovine Arp2/3 complex was a gift from T. Pollard (Yale University, New Haven, CT), and recombinant human Arp2/3 complex was a gift from M. Welch (University of California, Berkeley, Berkeley, CA). N-WASP VCA and ScarVCA were expressed in *E. coli* and purified as described previously (Zalevsky et al., 2001).

2D electrophoresis

The Arp2/3 complex was precipitated by chloroform/methanol extraction and resuspended in first-dimension rehydration buffer (8 M urea, 2% CHAPS, 0.5% pharmalyte, pH 3–10, 0.002% bromophenol blue, and 18 mM DTT). Protein samples were separated in the first dimension with an Ettan IGphor 3 IEF system (GE Healthcare) on Immobiline Dry Strips (pH 3–10NL, 7 cm; GE Healthcare) at 1,500 volt hours for 10 h. The focused proteins were then separated in the second dimension on Nu-PAGE 12% Bis-Tris precast gels (Invitrogen).

SDS-PAGE and immunoblotting

Arp2/3 complex was separated on 12.5% SDS-PAGE gels and transferred to Immobilon-P (Millipore) by standard methods. Immunoblots were incubated in 3% BSA in TBS-T (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 2 h, and monoclonal anti-pTyr (Cell Signaling Technology) or polyclonal anti-pThr (Invitrogen) were added at 200 ng/ml. Primary antibodies were detected with horseradish peroxidase–conjugated anti–mouse and anti–rabbit antibodies (BioRad Laboratories), respectively, and visualized with an ECL chemiluminescence kit (Pierce).

Arp2/3 complex dephosphorylation

Arp2/3 complex was dephosphorylated with AP (New England Biolabs, Inc.). Arp2/3 complex was diluted 1:1 with 2 mM Tris, pH 8, and combined with HipH Buffer (50 mM Tris, pH 8, 1 mM Mg_2Cl_2, and 0.1 mM $ZnCl_2$) containing 1 U AP and incubated at 30°C for 1.5 h. For mock-treated

controls, Arp2/3 complex was incubated with heat-inactivated phosphatase (65°C for 30 min) in HipH reaction buffer. For YOP dephosphorylation, Arp2/3 complex was incubated with 1 U YOP (New England Biolabs, Inc.) in 50 mM Tris-HCl, pH 7, 100 mM NaCl, 5 mM DTT, and 0.01% Brij 35 at 30°C for 1 h. For PP2Cα dephosphorylation, the Arp2/3 complex was incubated with 1 U PP2Cα (EMD) in 25 mM MES, 50 mM NaCl, 2 mM $MnCl_2$, and 1 mM DTT at 30°C for 1 h. The Arp2/3 complex was affinity purified after phosphatase treatment by using N-WASP VCA coupled to activated CH–sepharose 4B (GE Healthcare).

Actin polymerization

Pyrene actin polymerization assays were performed with 4 µM monomeric actin containing 5% pyrene-labeled actin in KMEI (50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, and 10 mM imidazole, pH 7), 5 nM Arp2/3 complex, and 500 nM ScarVCA/N-WASP VCA domain. Ca-ATP actin was converted to Mg-ATP actin by incubation in ME (50 mM $MgCl_2$ and 0.2 mM EGTA) before adding other assay components. Pyrene actin was excited in an RF-5301PC spectrophotometer (Shimadzu) at 365 nm, and fluorescence was measured at 407 nm at 1-s intervals. Actin filament barbed ends were calculated as described previously (Higgs, et al., 1999). Pointed elongation from gelsolin-capped actin filaments was measured as described previously (Mullins et al., 1998). Gelsolin-capped actin filaments (100 nM) were used for pointed end–binding assays with untreated and dephosphorylated Arp2/3 complex.

Quantification of Arp2/3 complex binding constants

Binding constants of Arp2/3 complex for NPFs were determined by using GST-ScarVCA and GST-N-WASP VCA covalently coupled to activated CH–sepharose 4B (GE Healthcare). GST-NPF–coupled beads were added to mock-treated or AP-treated Arp2/3 complex and incubated at room temperature for 30 min. NPF-coupled beads were spun at 700 *g* for 5 min, the supernatant was removed, and beads were resuspended in SDS-PAGE sample buffer. Coomassie-stained gels were scanned and quantified using a LabWorks imaging system and LabWorks Software (UVI). The data were plotted and fitted using GraphPad Prism software (GraphPad Software, Inc.). Binding constants for Arp2/3 complex for actin filaments were determined by actin cosedimentation as described previously (Mullins et al., 1997).

Mass spectrometry

Proteins were separated on 12.5% SDS-PAGE gels, individual bands were excised, and gel slices were destained with 25 mM NH_4HCO_3/50% acetonitrile. After reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide, proteins were digested with 12.5 ng/µl of recombinant porcine trypsin (Roche) in 25 mM NH_4HCO_3 at 37°C for 16 h. Peptides were extracted from the gel slices and cleaned using a $ZipTip_{C18}$ (Millipore) pipette tip. Mass spectrometry was performed with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Voyager-DE STR; Applied Biosystems).

MTLn3 cell culture and metabolic labeling

MTLn3 rat adenocarcinoma cells (provided by J. Condeelis, Albert Einstein College of Medicine, New York, NY) were maintained as described previously (Segall et al., 1996). For metabolic labeling, 3×10^6 cells were plated on 150-mm dishes and grown to ~85% confluence in MEMα without nucleosides supplemented with 10% FBS at 37°C, 5% CO_2. Cells were transferred to phosphate-free MEMα without nucleosides supplemented with 0.2 mCi/ml [^{32}P]orthophosphate for 4 h and then stimulated with 10 nM EGF. Cells were lysed at the indicated times with modified RIPA buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 1% NP-40, 1 mg/ml aprotinin, 1 mM pefabloc, and 1 mg/ml leupeptin) containing phosphatase inhibitors (1 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate), and the Arp2/3 complex was immunoprecipitated with anti-ARPC1 antibody (a gift from M. Welch, University of California, Berkeley) as described previously (Machesky and Insall, 1998).

S2 cell culture, Arp2 RNAi, and Arp2 mutants

D. melanogaster Schneider S2 cells were cultured as described previously (Rogers et al., 2002). Arp2 siRNA was performed with dsRNA to the 3′ untranslated region (UTR) coding sequence of *D. melanogaster* Arp2 (5′-GUGUGUGUGCGGACCGCAAGAAAUAGGAUAAAAAAGUGAUAGAUUUCUUUUCUCUAUUUUCUATAGGUUUAAACCUUUCAGAUUUACGUGAUAUAUCCGUCUAUAUAUGUUUUUUUUUU-3′). The Arp2 3′ UTR was cloned into a TOPO plasmid (Invitrogen) containing a T7 promoter. RNA was amplified using a T7 Megascript RNA amplification kit (Ambion). Plasmids expressing *D. melanogaster* Arp2 were constructed using Gateway cloning technology (Invitrogen). *D. melanogaster* Arp2 was cloned from an S2 cDNA library, sequenced, and inserted into a pENTR-D-TOPO plasmid (Invitrogen). Arp2 was then cloned into an expression vector containing a single C-terminal GFP. Mutant Arp2 sequences were constructed using a QuikChange Mutagenesis kit (Stratagene). RNAi was performed over 7 d as described by Rogers et al. (2003). In brief, 5 µg Arp2 double-stranded RNA was added to 24-well plates containing 3×10^5 cells on day one and again on day three. Cells were transformed with plasmids using Cellfectin LTX (Invitrogen) on day five. Plasmid transfection in S2 cells was ~30% efficient. On day seven, S2 cells were prepared for microscopy as described previously (Rogers et al., 2003) and mounted in fluorescent mounting medium (Dako). Images were acquired with a Roper SPOT charge-coupled device camera (Roper Scientific) on an Axiophot microscope (Carl Zeiss, Inc.) at 40× magnification at room temperature with Roper SPOT acquisition software. Images were assembled into figures using Photoshop (Adobe).

Arp2/3 structural model

Arp2/3 complex structure was analyzed using PyMol molecular viewing software and PDB 1K8K (Robinson et al., 2001). Images were imported and notated using Illustrator software (Adobe).

Online supplemental material

Fig. S1 shows data related to AP treatment of Arp2/3 complex with mock-treated samples, N-WASP VCA, and phosphatase inhibitors. Fig. S2 shows the effect of AP treatment on Arp2/3 complex subunit association, NPF-binding, and F-actin binding. Fig. S3 shows mass spectrometry data for phospho-Arp2 and immunoblots of Arp2-GFP from S2 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200802145/DC1.

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