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Arp2 depletion inhibits sheet-like protrusions but not linear protrusions of fibroblasts and lymphocytes

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Abstract

The Arp2/3 complex-mediated assembly and protrusion of a branched actin network at the leading edge occurs during cell migration, although some studies suggest it is not essential. In order to test the role of Arp2/3 complex in leading edge protrusion, Swiss 3T3 fibroblasts and Jurkat T cells were depleted of Arp2 and evaluated for defects in cell morphology and spreading efficiency. Arp2-depleted fibroblasts exhibit severe defects in formation of sheet-like protrusions at early time points of cell spreading, with sheet-like protrusions limited to regions along the length of linear protrusions. However, Arp2-depleted cells are able to spread fully after extended times. Similarly, Arp2-depleted Jurkat T lymphocytes exhibit defects in spreading on anti-CD3. Interphase Jurkats in suspension are covered with large ruffle structures, whereas mitotic Jurkats are covered by finger-like linear protrusions. Arp2-depleted Jurkats exhibit defects in ruffle assembly but not in assembly of mitotic linear protrusions. Similarly, Arp2-depletion has no effect on the highly dynamic linear protrusion of another suspended lymphocyte line. We conclude that Arp2/3 complex plays a significant role in assembly of sheet-like protrusions, especially during early stages of cell spreading, but is not required for assembly of a variety of linear actin-based protrusions.

Keywords

actin; leading edge; lamellipodia; filopodia; microvilli; dendritic nucleation

Introduction

The first step in cell motility is extension of the “leading edge” plasma membrane in the direction of motion [Mitchison and Cramer 1996]. This leading edge protrusion is actin-dependent, and actin polymerization is thought to supply the protrusive force. A variety of findings spread over three decades suggest that at least two spatially and dynamically distinct actin networks exist in the protrusive region of a cell’s periphery [Chhabra and Higgs 2007]. Classic studies by Abercrombie showed that the distal 1–5 microns, termed the “lamellipodium”, was fundamentally different from the bulk of the protrusive region, termed the “lamellum” [Abercrombie *et al.* 1970a; Abercrombie *et al.* 1970b; Abercrombie *et al.* 1970c; Abercrombie *et al.* 1971; Chhabra and Higgs 2007]. The lamellipodium is thinner, does not contain organelles, stains more densely in EM images, and is more dynamic in its protrusion/retraction. Recently, quantitative fluorescent speckle microscopy (qFSM) has shown that the lamellipodium predominantly contains highly dynamic, short-lived filaments, whereas the lamellum contains less dynamic, longer-lived filaments [Ponti *et al.* 2004].

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While lamellipodial filaments terminate abruptly along a band where stable adhesions first appear, lamellar filaments can be nucleated in the lamellipodium, but get more prominent beyond the lamellipodial band. Recent work by Giannone *et al.* [2007] suggests the lamellar network might spatially exist not only behind, but also beneath the lamellipodial network, potentially explaining the overlapping but distinct filament dynamics.

The molecular components of these two networks also appear to be distinct. Arp2/3 complex [Svitkina and Borisy 1999], cofilin [DesMarais *et al.* 2005], and capping protein [Mejillano *et al.* 2004] are more abundant in the lamellipodium [Iwasa and Mullins 2007; Lai *et al.* 2008], whereas certain tropomyosin isoforms [DesMarais *et al.* 2002; Iwasa and Mullins 2007], and non-muscle myosin [Verkhovsky *et al.* 1995] are more abundant in the lamellum. Electron micrographs of the leading edge suggest that the lamellipodium is composed of a dense network of short, branched filaments, while the lamellum contains a less dense network of longer, largely un-branched filaments [Svitkina and Borisy 1999]. Branched filament networks are characteristic of Arp2/3 complex activity [Goley and Welch 2006] and suggest a role for Arp2/3 complex in lamellipodial assembly, although some question the importance of dendritically branched networks at the leading edge [Koestler *et al.* 2008].

Biochemically, Arp2/3 complex facilitates assembly of actin branches by nucleating a new filament off the side of a pre-existing filament. Although Arp2/3 complex alone is inactive, activation can be induced by a nucleation promoting factor (NPF), such as WASp/Scar proteins, coupled with side-binding of the complex to an existing filament [Goley and Welch 2006]. By immunofluorescence, Arp2/3 complex is localized to the most distal portions of the leading edge [Kelleher *et al.* 1995; Machesky *et al.* 1997]. Immuno-gold EM of Arp2/3 complex demonstrates that Arp2/3 complex localizes to the actin filament branch point within lamellipodia [Svitkina and Borisy 1999]. qFSM studies show that Arp2/3 complex remains associated with the lamellipodial network through the majority of the network lifespan [Iwasa and Mullins 2007; Lai *et al.* 2008].

A number of groups have examined the role of Arp2/3 complex in lamellipodial assembly through the use of the Arp2/3 complex inhibitor N-WASP-CA [Gupton *et al.* 2005; Shao *et al.* 2006; Strasser *et al.* 2004], and through siRNA and shRNA targeted against Arp2/3 complex subunits [Di Nardo *et al.* 2005; Gomez *et al.* 2007; Rogers *et al.* 2003; Sarmiento *et al.* 2008; Steffen *et al.* 2006]. The results of these studies have been conflicting. Two groups have demonstrated that loss of Arp2/3 complex components has little or no effect on general cell morphology, motility or viability [Di Nardo *et al.* 2005; Sidani *et al.* 2007]. Specifically, one study found that mouse embryonic fibroblasts stably suppressed for Arp3 had minimal defects in migration and spreading [Di Nardo *et al.* 2005]. These results directly challenge the presumed role of Arp2/3 complex in leading edge protrusion and lamellipodial assembly.

In addition to the lamellipodial and lamellar networks, the leading edge of many cells contains filopodia, linear protrusions composed of tightly-bundled, un-branched actin filaments [Faix and Rottner 2006; Mallavarapu and Mitchison 1999]. Filopodia are often found interspersed within the leading edge lamellipodia and lamella and are characterized by the presence of fascin [Vignjevic *et al.* 2006], VASP [Schirenbeck *et al.* 2006; Svitkina *et al.* 2003] and myosin X [Bohil *et al.* 2006]. A number of different models have been proposed to explain their assembly. The convergent elongation model [Svitkina *et al.* 2003] describes the assembly of filopodia from the Arp2/3 complex nucleated dendritic network. In this model, nascent Arp2/3 complex-nucleated branches are protected from capping protein by the presence of a tip complex, which allows filaments to elongate while also undergoing bundling by fascin and other cross-linking proteins. This model is supported by both *in vitro* [Haviv *et al.* 2006; Vignjevic *et al.* 2003] and *in vivo* data [Korobova and

Svitkina 2008; Svitkina *et al.* 2003]. However, evidence suggests Arp2/3-independent mechanisms for filopodia assembly also exist [Ellis and Mellor 2000; Medalia *et al.* 2007; Pellegrin and Mellor 2005; Peng *et al.* 2003; Sigal *et al.* 2007; Steffen *et al.* 2006].

Microvilli are another type of linear protrusion, examples being brush border microvilli of epithelial cells [Mooseker and Tilney 1975; Tyska *et al.* 2005] and stereocilia of hair cells [Lin *et al.* 2005; Sekerkova *et al.* 2006]. Much shorter and highly dynamic microvilli are present on the surfaces of most [Gorelik *et al.* 2003], including lymphocytes [Majstoravich *et al.* 2004].

In this study, we utilize shRNA-mediated depletion of the Arp2 subunit to evaluate the role of Arp2/3 complex in assembly of two types of protrusive actin filament dependent structures. We first evaluate assembly of “sheet-like protrusions” at the edges of spreading cells, as well as on the surface of cells in suspension. We choose the term “sheet-like protrusion” rather than the common terms “lamellipodia” and “lamella” in an effort to remain objective about the effects of Arp2/3 complex suppression on protrusion morphology and dynamics. We also evaluate effects of Arp2/3 complex depletion on assembly of “linear protrusions” localized within the leading edge of spreading cells as well as on the surface of suspended lymphocytes. Although these linear structures might be termed “filopodia” or “microvilli”, we choose a less-specific term because the composition and origin of these structures is difficult to define.

We demonstrate that Arp2-depletion inhibits sheet-like protrusion and the initial spreading ability of fibroblasts during replating, but does not eliminate spreading ability over an extended time course. Similarly, Arp2-depletion in Jurkat T cells inhibits not only spreading of sheet-like structures during cell spreading on anti-CD3, but also sheet-like ruffle structures on the surface of suspended cells. In contrast, Arp2-depletion has no effect on assembly of three different types of linear protrusion including 1) constitutive linear protrusions on the surface of a cultured lymphocyte line; 2) transient linear structures on the surface of mitotic Jurkats; or 3) linear structures around the periphery of both spreading fibroblasts and Jurkats. Together, these results indicate that Arp2/3 complex plays a role in spreading and initial leading edge sheet-like protrusions, but is not necessary for assembly of a variety of linear protrusions.

Methods

Cells and reagents

Murine Swiss 3T3 fibroblasts were grown in DMEM supplemented with 10% calf serum (Atlanta Biologicals) and penicillin/streptomycin and maintained at <80% confluency. Human Jurkat T lymphocytes were grown in RPMI 1640 (Gibco) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals), 2mg/ml glucose, 1mM sodium pyruvate (Gibco), and 50μM β-mercaptoethanol (Gibco), and maintained in suspension at concentrations <1 million cells/ml. Murine 300.19 pre-B lymphoma cells were grown in RPMI supplemented with 5% FBS and 50μM β-mercaptoethanol, and maintained in suspension at concentrations <1 million cells/ml.

Polyclonal rabbit anti-Arp2 (H-84, sc-15389; Santa Cruz Biotechnology, Inc) and anti-p34 (Upstate) were used to detect Arp2/3 complex by western blot. Mouse monoclonal anti-β-actin (c4D6; Upstate) was used to evaluate total actin levels by western blot. fluorescence microscopy, TRITC-phalloidin (Sigma) was used to detect polymerized actin, mouse monoclonal anti-tubulin (DM1α; Sigma) was used to detect microtubules and DAPI was used to detect nuclei. Goat anti-Rabbit HRP (Invitrogen), Goat anti-Mouse HRP (Invitrogen) and Alexa Fluor 680 rabbit anti-mouse (Molecular Probes) secondary antibodies were used

for western blot and immunofluorescence, respectively. Poly-L-lysine (>300,000 MW) and fibronectin for spreading assays were purchased from Sigma and BD Biosciences.

Constructs

To create an anti-Arp2 shRNA plasmid, GFP.pSuperior.retro.neo control shRNA vector (OligoEngine) was digested with BglII/HindIII. Double-stranded shRNA hairpin inserts targeting human Arp2 coding sequence were then ligated into the vector to create “GFP-anti-Arp2 shRNA”. The target sequence for Arp2 (nt 981–999) was based on the sequence published by Komano *et al.* [2004] (ACAGCTTTACTTAGAACGA), which is 100% identical to the murine Arp2 (nt 965–983). pEGFP- β -Actin was kindly provided by Clare Waterman-Storer. GFP-Arp3 was purchased from Addgene.

Transfections

For 3T3 cell transfections, cells were trypsinized, washed twice with DPBS (with Ca^{++} & Mg^{++}), then re-suspended in DPBS (with Ca^{++} & Mg^{++}). Cells were electroporated in BTX-40 4mm gap cuvettes (Fisher) using the GenePulser II (BioRad) set for 300V, 0.975 Capacitance. For expression of GFP-fusion proteins, 10–20 μg of plasmid was transfected into 2–5 million cells per cuvette, then cells were evaluated after 24–48 hours. For knockdown experiments, 50 μg GFP-pSuperior (empty vector control) or GFP-anti-Arp2 shRNA plasmid was transfected into 1.8–2 million cells per cuvette.

For Jurkat transfections, cells were washed and re-suspended in DPBS (with Ca^{++} & Mg^{++}), then electroporated at 280V at 0.975 Capacitance. Expression of GFP-fusion proteins required 10–20 μg plasmid per 5 million cells whereas knockdown experiments required 50 μg plasmid per 5 million cells.

For Arp2-depletion in 300.19 cells, 2 million cells were washed and re-suspended in DPBS (with Ca^{++} & Mg^{++}), then electroporated at 250V, 0.975 capacitance, using 50 μg control or shRNA plasmid. Because 300.19 cells divide so rapidly, cells were re-transfected after 30 hours following the same protocol as first transfection.

Evaluation of Arp2-knockdown by immunoblot

To determine timing for optimal knockdown efficiency, cells were transfected, then evaluated by flow cytometry for GFP levels and by western blot for Arp2 protein levels after 24, 48, 72, and 96 hours. Prior to flow cytometric analysis of 3T3s, cells were washed, trypsinized, centrifuged 10 min at 1250 RPM, then suspended in PBS. Jurkats and 300.19 cells were washed in PBS, centrifuged 5 min at 1300 RPM, then re-suspended in PBS. Transfected cells were evaluated live for GFP-expression levels using a FACScan (BD Biosciences).

For cell extract preparation, equal numbers of cells were taken from either the empty vector control or the anti-Arp2-shRNA transfected population. Cells were then divided between two aliquots, one for Bradford Assay and the other for cell extract. For Bradford Assay, cells were lysed in PBS with 1% thesitol, then assayed according to manufacturer's protocol (BioRad). For extracts, cells were lysed in Lysis Buffer (50mM HEPES pH 7.5, 4%SDS, 300mM NaCl, 1mM EDTA, 0.1mM DTPA, 5 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatinA, 1 $\mu\text{g}/\text{ml}$ antipain, 5 $\mu\text{g}/\text{ml}$ E-64, 0.5 $\mu\text{g}/\text{ml}$ calpeptin, 1 $\mu\text{g}/\text{ml}$ calpain inhibitor 1, 0.05 $\mu\text{g}/\text{ml}$ cathepsin B inhibitor II, 1mM DTT), boiled 10 minutes, cooled, then treated with 300nM N-ethylmaleimide and stored at room temperature. Equal concentrations of extract from control and Arp2-depleted cells were analyzed by western blot using anti-Arp2, anti-p34 and anti-actin antibodies. Densitometry analysis of western blots was carried out using ImageJ.

Arp2/3 complex quantification in Swiss 3T3 cells

Quantitative western blotting to determine cellular concentration of endogenous Arp2/3 complex was done following procedure of Higgs *et al.*[1999]. In short, extracts were prepared in same manner as for evaluation of knockdown, with only alteration being inclusion of extra aliquot of cells for quantifying concentration of cells. Quantitative western blotting was performed by running equal concentrations of total extract on gel with varied concentrations of purified bovine Arp2/3 complex spiked into extract, then calculating Arp2 concentration from densitometric analysis of blots. By Bradford analysis of total cell extract, trypsinized 3T3 cells contain 0.7ng total protein/cell. Mean cellular volume (2.85×10^{-12} L) and nuclear volume (0.59×10^{-12} L) were calculated from mean diameters of trypsinized 3T3 cells (17.6 μ m) and nuclei (10.4 μ m), from rhodamine-phalloidin and DAPI stained images of trypsinized cells, respectively ($n > 30$ cells). Western blot signal was linear for cell extract from 1–4 μ g extract, and from 0.2–1 ng purified bovine Arp2/3 complex. We used 2 μ g extract for concentration determination.

Fibroblast Spreading Assay

Acid-washed glass coverslips were coated with 0.01% PLL (diluted in sterile water) or 10 μ g/ml fibronectin (diluted in sterile water) for at least 2 hours. Just prior to use, coverslips were washed once with water and dried for 5 minutes. 3T3 cells were trypsinized, washed twice with room temp PBS, and centrifuged in a swinging bucket rotor at $246 \times g$ for 10 min between each wash. Cells were then re-suspended in pre-warmed medium and diluted to appx. 80–100,000 cells/ml.

Jurkat Spreading Assay

Acid-washed coverslips were washed once in 70% EtOH, then dried in hood. Coverslips were then coated with 0.1% PLL (in water) for 5 minutes, washed with water, then dried. Coverslips were then transferred to 24 well plate, then coated with 280 μ l anti-CD3 (BD Pharmingen) diluted to 10 μ g/ml in sterile PBS for at least 2 hours at 37 degrees. Just prior to use, coverslips were washed once with PBS. Jurkats were washed once in PBS, then re-suspended to 2 million cells/ml. in medium. For SEM and light microscopy, 1ml cells were transferred to each coverslip, then allowed to spread for 5 minutes prior to fixation. For live cell imaging, an anti-CD3 coated coverslip was dabbed onto kimwipe then quickly adhered to the base of a modified Rose chamber using VALAP. The chamber was filled with medium alone, and assembled on microscope prior to adding cells. To begin spreading assay, medium was removed and 50ul cells were added to coverslip and allowed to spread during live imaging.

Immunofluorescence and Electron Microscopy

For immunofluorescent imaging of polymerized actin and tubulin, cells were fixed for at least one hour in 4% formaldehyde, 0.1% saponin, 0.05% glutaraldehyde and $1 \times$ PBS. Spread 3T3 cells and Jurkats were fixed directly on coverslip. Jurkats and 300 cells fixed in suspension were first adhered to coverslips by washing once in PBS, re-suspending in PBS (100 μ l/coverslip) then allowing to settle onto coverslips pre-coated with 0.1% PLL. After settling for 20 minutes, cells were fixed to coverslip using 0.5ml immunofluorescent fix. Coverslips were washed once with PBS, treated $3 \times$ for 8 minutes with fresh sodium borohydride (0.2% in water), washed again with PBS, then blocked for 30 minutes (10% calf serum, 0.1% saponin, 0.02% sodium azide in PBS). After block, coverslips were stained for 1 hour with primary antibody or rhodamine-phalloidin (0.1 μ M), washed with PBS, blocked for 15 minutes, then stained for 1 hour with secondary antibody. To stain nuclei, DAPI was added during the last 5 minutes of secondary stain. After staining, coverslips were washed $3 \times$ with PBS, then adhered to slides using PVA-DABCO. Slides were dried

overnight, then rinsed with water prior to viewing. Cells were imaged using a Nikon TE2000-E inverted microscope and Roper CoolSnap camera. Images were acquired using Metaview (Universal Imaging Corp) or Nikon NIS software.

For SEM, cells were fixed for 1–2 hours in 3.5% glutaraldehyde in 100mM NaPO₄, pH 7.4. Suspension cells were adhered to PLL-coated coverslips in same manner as for immunofluorescence. Coverslips were washed with 20mM sodium phosphate (pH 7.5), then stained for 1 hour with 1% osmium tetroxide (Electron Microscopy Sciences) diluted in 100mM sodium phosphate (pH 7.5). Coverslips were washed once, then dehydrated sequentially with 30, 50, 70, 95, and 100% ethanol. Coverslips were washed 2× for 5 minutes with 1:1 and 1:2 dilutions of Ethanol:Hexamethyldisilazane (HMDS; Sigma), then 3× for 20 minutes with 100% HMDS. Coverslips were dried overnight under vacuum in a desiccator. Prior to viewing, coverslips were adhered to aluminum SEM stubs, then coated with 3nm osmium tetroxide using an OPC-60 Osmium Plasma Coater (SPI Supplies). Cells were imaged in SE mode with an FEI XL-30 FEG ESEM using an accelerating voltage of 5kV, spot size 3.

Evaluation of Arp2-knockdown by immunofluorescence

For immunofluorescent staining of Arp2, cells were fixed with 4% formaldehyde, 0.1% saponin, 1× PBS for 15 minutes. Cells were then washed 1× with 1× PBS, then extracted for 15 minutes with ice-cold methanol. Cells were re-hydrated for 15 minutes in 1× PBS, then prepared for staining using same protocol as for rhodamine-phalloidin and tubulin staining (see above). For evaluation of knockdown, cells were selected using a round region of interest (ROI) tool with our Nikon NIS software, then measured for total and mean average fluorescence intensity. In order to partially correct for background fluorescence, ROIs of equal size that lacked cells were also measured and these values were subtracted from the measured values of control and Arp2-depleted cells.

Live Cell Imaging

For live cell imaging of 3T3 spreading, modified Rose chambers [Rieder and Cole 1998] were assembled by first adhering a PLL-coated coverslip to the base of the chamber using VALAP. Cells were added to the chamber, then the chamber was sealed with an uncoated coverslip and VALAP. Cells were incubated at 37°C, 5% carbon dioxide for 15 minutes, then transferred to the microscope room adjusted to 32°C. GFP+ cells were located and selected for imaging. Time lapse images of cells were taken every 30 seconds. For imaging of Jurkat spreading, modified Rose chambers were assembled with an anti-CD3 coated coverslip at the base of the chamber, adhered using VALAP. Chamber well was filled with medium while chamber was placed on microscope and focused on coverslip surface. Medium was removed and cells were added to chamber. As soon as GFP+ cells were located, time lapse images were taken every 20 seconds as the cell spread onto the coverslip.

Image Analysis

Images were collected using Metaview and Nikon NIS software. Images for manuscript were processed using ImageJ and Photoshop. Brightness and contrast were adjusted independently for each channel to optimally demonstrate cell morphology or cytoskeletal structures. Protrusion dynamics were measured from frame-by-frame comparison of time lapse images. Spreading efficiency was quantified by visual assessment of nuclear flattening. By immunofluorescence, nuclei of well-spread cells were in focus in a plane near the basal cell surface and adhered protrusions. Analysis of the percent dynamic periphery of stably adhered cells was completed by tracing and measuring the total cell periphery. Time-lapse sequences of cells were then used to determine which regions of cell were dynamic and these regions were then traced and measured. The sum of the dynamic peripheral

regions divided by the total periphery was used to determine the percent dynamic edge of stably adhered cells. Averages and SEM were calculated using Microsoft Excel.

Results

Arp2-depletion alters protrusion, but not morphology, of stably adhered fibroblasts

Swiss 3T3 fibroblasts are highly adherent cultured cells that have been used in many studies of actin dynamics and regulation. Cells were transfected with GFP-anti-Arp2 shRNA plasmid or the paired GFP-Empty Vector control, then evaluated for GFP expression and Arp2 levels at various time points after transfection. Cells transfected with the GFP-pSuperior Empty Vector or the GFP-anti-Arp2 shRNA construct are henceforth referred to as “control” or “Arp2-depleted”, respectively. We found that Arp2 depletion was optimal between 78–84 hours after transfection (data not shown). Flow cytometry of GFP-levels, as a measurement of transfection efficiency, indicated an average of 18% GFP+ cells after 78 hours. Western blots of cell extracts indicate an average of 34% decrease of Arp2 levels in the unsorted population of anti-Arp2 shRNA-transfected cells (Fig. 1a). Because the level of knockdown exceeds the percentage of GFP+ cells, we infer that Arp2-suppression in GFP+ cells is high in GFP+ cells and that other cells with GFP levels below the detection limit of flow cytometry also exhibit Arp2-depletion. Immunofluorescence staining of Arp2 in GFP+ control and Arp2-depleted cells demonstrates an average 72% decrease in total fluorescence intensity, confirming depletion of Arp2 in targeted cells (Figure 1B). We believe this value to be an under-estimate of knock-down efficiency, due to high background in the Arp2 staining.

We first examined effects of Arp2-depletion on the actin organization and general morphology of cells stably adhered to coverslips, identifying transfected cells by GFP-fluorescence. Fixed cells were stained for polymerized actin and tubulin. Control fibroblasts that have been plated for extended times (greater than 8 hours) exhibit a stretched, and strongly adhered morphology. Similar to results reported by Di Nardo *et al.* [2005], Arp2-depleted fibroblasts fixed at 78 hours exhibit no obvious difference in morphology or overall actin organization at the cortex or in stress fibers compared to control cells (Fig. 1C,D). When cells plated for extended times are observed live, 28% of the periphery of control cells, generally focused at cell “corners” (Fig. 1E,F), exhibits dynamic protrusion and retraction. Similarly, 33% of the periphery of Arp2-depleted cells exhibits active protrusion and retraction. Although Arp2-depleted cells exhibit a similar shape in fixed images as well as similar percentages of dynamic edges, time-lapse imaging reveals that their sheet-like structures exhibit a defect in protrusive ability (Fig. 1E–H). Protrusion rates of sheet-like protrusions in Arp2-depleted cells are 45% slower than those in control cells (Fig. 1G–H, Table 1; Movie S1, S2). Our results indicate that even minimal Arp2/3 complex expression is sufficient for extension of small protrusions around limited regions of the cell periphery.

Arp2-depletion results in defects in 3T3 spreading upon replating

To test effects of Arp2-depletion on more dynamic actin protrusions, we employed a spreading assay of 3T3 cell replating onto poly-L-lysine (PLL) coated coverslips. Under this regimen, cells spread following a predictable time course, as demonstrated by images of cells fixed for both Scanning Electron Microscopy (SEM, Fig. 2A–C) and immunofluorescence (Fig. 2D–F) at different time points after replating. By 15–20 minutes, cells adhere, and form sheet-like protrusions that are parallel to the coverslip, and are uniformly extended around the cell periphery (Fig. 2A, D). Between 20–60 minutes, cells achieve a more flattened morphology, in which more pronounced peripheral protrusions are noticeable (Fig. 2B, E). Many cells apparently undergo a break in symmetry within the first hour, as evidenced by a retracted edge on one side of a cell accompanied by one or more

sheet-like protrusions at an opposite edge. By one hour, cells exhibit a highly-digitated morphology (Fig. 2B, E) and by 8 hours, cells exhibit a fully-extended, flattened morphology (Fig. 2C, F) with abundant stress fibers.

Live cell imaging of cells expressing GFP-actin (Fig. 2G, Movie S3) or GFP-Arp3 (Fig. 2H, Movie S4), confirms the spreading process that we observe for fixed cells. GFP-actin localization within both the nascent sheet-like (Fig. 2G) and linear protrusions (data not shown) is clearly visible as the cell spreads. Similarly, GFP-Arp3 (Fig. 2H) shows clear localization to the leading edge of a cell spreading at a slightly later time course, which supports published immunolocalization of Arp2/3 complex to lamellipodial protrusions [Welch *et al.* 1997], [Svitkina & Borisy 1999]. Notably, we find no GFP-Arp3-positive linear protrusions within the sheet-like structures (Fig. 2H, data not shown), consistent with staining results in other cell types [Svitkina and Borisy 1999].

We next evaluated Arp2-depleted fibroblasts for defects in replating and spreading proficiency. After 20 minutes on PLL, fixed GFP+ control cells exhibit similar spreading morphology to untransfected control cells, with uniform sheet-like protrusions around their periphery (Fig. 3A). Approximately 93% of control cells exhibit either smooth (71%) or ruffled (22%) peripheral edges (Fig. 3A,C). In contrast, Arp2-depleted cells exhibit severe spreading defects (Fig. 3B) with more than 90% of Arp2-depleted cells exhibiting a stellate, “spiky” morphology (Fig. 3C) somewhat resemblant of peripheral filopodia. The stellate morphology of our Arp2-depleted fibroblasts resembles that of *Drosophila* S2 cells depleted of the p34 subunit of Arp2/3 complex [Rogers *et al.* 2003]. As Arp2-depleted cells continue to “spread” and flatten onto the coverslip, these filopodia-like protrusions increase in width and sometimes exhibit small sheet-like or ruffle-like protrusive structures along their length (Fig. 3B, 120 min). When allowed to spread for extended times (8 hours), Arp2-depleted cells eventually achieve a more “normal”, spread morphology, similar to that of control cells (bottom panels, Fig. 3A,B). When evaluated between the 20 and 60 minute spreading time points, Arp2-depleted cells exhibit a 35% average decrease in spreading efficiency (Fig. 3D), defined by a cell’s ability to achieve nuclear flattening. These results indicate Arp2/3 complex plays a major role in the initial spreading response during fibroblast replating, but that cells are still capable of achieving a flattened and spread morphology after extended times. We also evaluated cell spreading on un-coated coverslips and fibronectin-coated glass coverslips (Supp. Fig. 1). For all observed substrates, Arp2-depleted cells exhibit a stellate, spiky morphology at early spreading time points (Supp. Fig. 1B,D,F).

From the fixed cell images, we could infer a loss of sheet protrusions in the Arp2-depleted cells, but we could not definitively determine whether this was because 1) sheet protrusions never initiated, 2) sheet assembly was delayed, or 3) sheet protrusions were extremely unstable and exhibited rapid retraction. In order to address the nature of sheet like protrusion and retraction in our Arp2-depleted cells, we utilized phase contrast time lapse imaging (Fig. 4). We first compared GFP+ and GFP- cells within the same anti-Arp2 transfected population (Fig. 4A–E; Movies S5, S6). With the caveat that some GFP- cells probably exhibit reduced Arp2/3 complex levels, we still observe a dramatic distinction in spreading behaviors between the two populations of cells. We then compared GFP+ Arp2-depleted cells with GFP+ empty vector control cells (Fig. 4F–I; Movies S7, S8). In both cases, control cells exhibit rapid sheet extension after adhesion to the coverslip, followed by cycles of sheet protrusion and retraction. Protrusion rates of sheet-like protrusions in control cells averaged $1.65 \pm 0.35 \mu\text{m}/\text{min}$, a rate slower than that observed for sheet-like protrusion of stably adhered cells (Table I). Notably, linear fibers remain adhered to the coverslip after sheet retraction. These linear protrusions appear to provide the “rails” along which subsequent sheet re-protrusion occurs (Fig. 4C, H; Movies S6, S8).

In contrast, Arp2-depleted cells exhibit several key differences. First, many Arp2-depleted cells display long, linear adhesions. These structures are phase-dense and exhibit slow protrusion and retraction. Interestingly, it is only along the length of these linear protrusions that small sheet and ruffle-like protrusions extend and retract at an average rate of $0.53 \pm 0.05 \mu\text{m}/\text{min}$. (Fig. 4D, E; Movie S6). A sub-population of Arp2-depleted cells exhibit a less severe phenotype with uniform sheet protrusions that are not limited to the sides of the linear protrusive structures. (Fig 4H, I; Movie S8). The rate of sheet-protrusion in both Arp2-deficient phenotypes is 68% slower than in control cells (Table 1; Fig. 4B, D, E, G & I).

Arp2-depleted Jurkats exhibit spreading defects on anti-CD3 coated coverslips

Jurkat T lymphocytes provide another unique system for studying actin dynamics. Jurkats can be stimulated to spread on anti-CD3 coated coverslips in a process similar to 3T3 cell spreading during replating, but without the trypsinization procedure required for 3T3s (Supp. Fig. 2A–C). This process is rapid, with spreading occurring within 2 minutes of cell addition to coverslips [Kaizuka *et al.* 2007]. Live cell imaging of cells expressing GFP-actin demonstrates that the outer edge of the spreading cells is highly dynamic, spreading in a manner similar to the sheet-like protrusions of 3T3 fibroblasts (Table 1; Supp. Fig. 2D, Movie S9). As the cell spreads, the dense actin band expands, then disperses (Supp. Fig. 2D) as the cell partially retracts from the coverslip (Supp. Fig. 2D, far right). Live cell imaging indicates that GFP-Arp3 is enriched at the spreading edge but not as strikingly as GFP-actin (Supp. Fig. 2E, Movie S10).

For Arp2 depletion, we transiently transfected Jurkats with either our GFP-pSuperior control vector or our GFP-anti-Arp2 shRNA construct, then evaluated for GFP expression (by flow cytometry) and Arp2 levels (by western blot) at various time points after transfection. Arp2-depletion in Jurkats was optimal 72–78 hours after transfection (data not shown). At 72 hours, GFP-expression levels average 65% (data not shown), while western blot of extracts indicates an average of 72% decrease of Arp2 (Supp. Fig. 2F). As with 3T3 cells, the reduction in Arp2 levels in Jurkats consistently exceeds the apparent percentage of GFP-positive cells, indicating that even cells with GFP levels below detection exhibit significant knockdown.

To evaluate effects of Arp2-depletion on anti-CD3-stimulated Jurkat spreading, we allowed control and Arp2-depleted cells to spread for 2–5 minutes on anti-CD3 coated coverslips, then fixed for SEM (Supp. Fig. 2G, I) or immunofluorescence (Supp. Fig. 2 H, J). Figure 5 shows SEM images of control (Supp. Fig. 2G,H) and Arp2-depleted (Supp. Fig. 2I,J) cells at various stages of spreading on anti-CD3. By SEM, we evaluated cells for changes in morphology, including: 1) smooth sheet-like peripheral protrusions; and 2) spiky, filopodia-like protrusions. Cells were also evaluated for spreading efficiency, or ability to “flatten” onto the coverslip. Similar to results reported by Gomez *et al.* [2007], control cells exhibit mostly smooth or ruffled peripheral protrusions (Fig. 5G), while Arp2-depleted cells predominantly exhibit spiky protrusions and stellate adhesions (Supp. Fig. 2I). Quantification of SEM-fixed cells indicated that Arp2-depleted cells exhibit a 27% increase in spiky peripheral protrusions and a 29% decrease in spreading efficiency (Supp. Fig. 2K; data not shown). Since we are unable to distinguish GFP+ cells by SEM, we are unable to determine whether the cells observed are truly Arp2-depleted.

Cells fixed for immunofluorescence were stained for polymerized actin (Supp. Fig. 2H, J), then evaluated for the presence of a dense, smooth actin band at the cell periphery. Arp2-depleted Jurkats exhibit a 40% decrease in cells with a smooth actin band (Supp. Fig. 2J, K), instead exhibiting spiky, actin-rich filopodia-like structures. In addition, the GFP+ Arp2-depleted cells exhibit nearly a 40% decrease in spreading efficiency (Supp. Fig. 2L). Taken

together, both the SEM and immunofluorescent data suggest that Arp2-depleted Jurkat cells are deficient for spreading on anti-CD3.

Arp2-depletion in Jurkats induces an increase in non-ruffle, microvillar surfaces

Jurkat T lymphocytes grown in suspension are covered on their surface by large actin-rich ruffle structures (Fig. 5A,B) which contain embedded actin striations, reminiscent of the linear protrusions embedded within the sheet-like structures in fibroblasts. Surface ruffles are commonly accompanied by short microvilli, but it is rare to find cell surfaces covered by microvilli alone. GFP-Arp3 localizes to ruffle structures, but does not label the linear striations (data not shown). Due to the small microvillar size, we were unable to determine whether GFP-Arp3 localized to these structures.

SEM evaluation of surface morphology indicated that 94% of control cells exhibit ruffles on their surface, and the remaining 6% of control cells exhibit either long microvilli (3.5%) or a bald or blebbed surface (2.5%) (Fig. 5, E; Fig. 6A, B). In contrast, only 81% of Arp2-depleted cells exhibit ruffles, while 13.5% of cells are covered with microvilli alone, and 5.5% cells exhibit a bald or blebbed surface (Fig. 5D, E). Quantitatively, the decrease in ruffles of Arp2-depleted cells seems quite modest. Qualitatively, however, the ruffles of Arp2-depleted appear considerably less developed and smaller, with a more ragged edge than control cells (Fig. 5C, D). For example, the cell in the middle panel of Fig. 5D was included as a cell exhibiting ruffles although these ruffles are not the predominant protrusion evident on the surface of this cell. Due to the heterogeneity of ruffles on control cells, we had difficulty in quantifying meaningfully this difference in ruffle morphology.

The microvilli of Arp2-depleted Jurkats are not a result of mitotic arrest

We were intrigued by the small but striking population (appx. 3.5%) of control suspension Jurkats exhibiting long microvilli (>1µm in length) and devoid of ruffles (Fig. 6F). These microvilli are visible not only by SEM (Fig. 6A) but also by fluorescence microscopy when stained for polymerized actin (Fig. 6B). All microvilli-covered Jurkats appear to be in some stage of mitosis, revealed by staining of DNA and microtubules (data not shown). To test the link between mitosis and the microvillar phenotype, we used low-concentrations of nocodazole (100ng/ml) to block cells in pro-metaphase. After 18–20 hours in nocodazole, cells were washed and re-suspended in fresh medium, then fixed for both SEM and immunofluorescence at various time points (Fig. 6A, B). At each time point, we analyzed cells by immunofluorescence for mitotic index (% cells in mitosis) and by SEM for surface morphology. By 45 minutes post-washout, 97.4% of untreated cells are in interphase, while 83.6% of the nocodazole-blocked cells are mitotic. Corresponding strongly with the mitotic index, 92.7% of untreated Jurkats exhibit a ruffled morphology while 3.6% of cells exhibit only microvilli; the remaining cells are either bald or blebbed (Fig. 6A, D). At 45 minutes post-nocodazole washout, 72.5% of the cells exhibit only microvilli on their surface (Fig. 6B–D). By 4 hours post-nocodazole washout, the mitotic index returns to the unsynchronized value, with approximately 91% of the cells in interphase and greater than 50% of cells regaining their ruffled phenotype (data not shown).

We asked whether the increase in microvilli-covered cells within the Arp2-depleted population was a secondary effect due to a mitotic block. Analysis of the mitotic index of our control and Arp2-depleted cells revealed no significant change in the mitotic index of the two cell populations (Fig. 6E). These results indicate that the increase in microvillar morphology is not simply due to an increase in mitotic cells and might instead be indicative of a defect in ruffle assembly. Moreover, mitotic GFP+ Arp2-depleted Jurkats still exhibited clear linear protrusions, visible by immunofluorescence imaging of the cell's surface, suggesting that Arp2-depletion does not affect these structures (data not shown).

Arp2-depletion has no effect on microvillar formation in 300.19 lymphoma cells

Our observations of the effects of Arp2-depletion on the mitotic microvilli of Jurkats suggest that Arp2/3 complex is not required for assembly of these linear structures. We tested the effects of Arp2-depletion on a second type of microvillar structure. Murine 300.19 pre-B lymphoma cells are covered on their surface by short, highly dynamic microvilli, similar to blood-borne, un-activated primary lymphocytes [Majstoravich *et al.* 2004]. Optimal Arp2-depletion in 300.19 cells is observed at appx. 66 hours post-transfection. GFP-expression levels at 65–70 hours range from 67–72% for both control and Arp2-depleted cells. Western blot of extracts demonstrates an average 65% decrease in Arp2 levels for the total population of anti-Arp2-shRNA transfected cells (Fig. 7A). Not only do we observe depletion of Arp2 in our total population of anti-Arp2-shRNA transfected cells, we also observe depletion of the p34 (ArpC2) subunit, suggesting that Arp2-depletion destabilizes other subunits in the Arp2/3 complex (Fig. 7A).

We fixed cells for both immunofluorescence and SEM and evaluated for actin organization and surface morphology. Because these microvilli are 300–400nm in length [Majstoravich *et al.* 2004], they are at the limit of resolution for the light microscope. Using a 100× 1.4 NA objective, when we focus on the top and bottom surfaces of cells, we observe small actin-rich puncta (Fig. 7B). We infer these puncta to be microvilli, based on comparison with SEM images of surface architecture. Cells also exhibit a band of actin filament staining around the peripheral edge in images taken at the middle of the cell (Fig. 7B, right). Arp2-depleted cells clearly exhibit actin-rich puncta by fluorescence microscopy (Fig. 7C), and microvilli by SEM (Fig. 7D, E). SEM images of control and Arp2-depleted cells are indistinguishable with appx. 96% and 97% of cells displaying microvilli on their surfaces, respectively (n>70 cells; data not shown). Actin filament staining reveals no apparent decrease in intensity of the actin-rich band at the cell periphery.

Discussion

Summary of Results

We provide evidence supporting the role of Arp2/3 complex in the spreading processes of two cell types, Swiss 3T3 fibroblasts and Jurkat T lymphocytes. Arp2-depleted 3T3 cells plated for extended times exhibit morphologies similar to control cells but the dynamics of protrusion are slower in these cells. More dramatically, the initial spreading process is severely compromised in Arp2-depleted cells. Moreover, initial protrusion in severe Arp2-depleted cells is limited to regions along the length of the linear protrusions.

Similarly, Jurkat T lymphocytes stimulated to spread on anti-CD3 coated coverslips exhibit defects in spreading ability coupled with formation of a stellate morphology. Arp2-depletion also induces a decrease in ruffles and an increase in microvillar and bald surfaces on unstimulated Jurkats in suspension. In contrast to the morphological effects of Arp2-depletion on the sheet-like structures, Arp2-depletion has no obvious effect on the mitotic microvilli of Jurkats or the constitutive microvilli of 300.19 pre-B lymphoma cells. Collectively, these results suggest that Arp2/3 complex plays an important role in the assembly of actin filaments in lamellipodial structures, but does not play a significant role in the formation and protrusion of linear protrusions such as filopodia and microvilli.

Reconciling Previous Data: Role of Arp2/3 Complex in Lamellipodial Protrusion

Although a number of groups have experimentally examined the role of Arp2/3 complex in lamellipodial assembly, the results have been conflicting. Most studies of Arp2/3 complex inhibition have yielded results indicating defects in lamellipodial protrusion. Steffen *et al.* [2006] demonstrated that depletion of the p21 or Arp3 subunits of the complex yielded loss

of lamellipodial formation in B16F1 mouse melanoma cells. Similarly, Kempia *et al.* [2003] demonstrated that depletion of p34 led to loss in protrusive ability of MTLn3 cells after EGF-stimulation. Knockdown of other proteins important to the dendritic nucleation model, including Scar/WAVE proteins [Rogers *et al.* 2003; Sarmiento *et al.* 2008] and capping protein [Mejillano *et al.* 2004] have similar effects.

In contrast to these results, work by Di Nardo *et al.* [2005] revealed that mouse embryonic fibroblasts (MEFs) with stable suppression of >90% Arp3 protein exhibited a stably adhered morphology and lamellipodial actin organization identical to control cells. Similarly, wound healing assays revealed no difference in overall migration rates. After PDGF stimulation, Arp3-depleted cells display a slight decrease in ruffle presence and lamellipodial protrusion rate. Similarly, these cells exhibit a modest decrease in spreading rates on fibronectin. From these collected data, the authors conclude that Arp2/3 complex is not required for leading edge protrusion [Di Nardo *et al.* 2005]. Similarly, Sidani *et al.* [2007] reported that knockdown of the p34 subunit in MTLn3 cells yielded morphologies and protrusion rates that were undistinguishable from control cells.

One of our objectives was to reconcile these opposing studies and identify possible explanations for the differences. Similar to the work by Di Nardo *et al.* [2005], we see no obvious defects in the overall morphology of stably adhered Arp2-depleted cells. However, time lapse imaging of these cells reveals a 45% decrease in sheet-like protrusion rates in the small percentage of the cell periphery that is dynamic. The more obvious defects in sheet-like protrusion are only observed during the replating assay, when Arp2-depleted cells are challenged to reorganize a considerably larger percentage of their peripheral actin cytoskeleton. During replating, control cells form uniform and highly dynamic sheet-like protrusions. Arp2-depleted cells exhibit a stellate-shaped morphology, and sheet-like protrusions are mostly limited to the lengths of large, linear protrusions. Because we do not know the detailed architecture of these linear protrusions, it is not clear whether they are filopodia-like, lamellar-like, or stress fiber-like structures. Interestingly, the residual sheet-like protrusions extending along the sides of the linear protrusions exhibit a 68% decrease in protrusion rates as compared with overall sheet-like protrusion rates in control cells. In spite of their slower spreading dynamics, Arp2-depleted cells are eventually able to achieve a fully spread morphology, comparable to control cells. We interpret these results to indicate that Arp2/3 complex plays a major role in the assembly of the large, uniform sheet-like structures formed during the initial spreading response, as well as the smaller, transient sheet-like protrusions formed at the corners of stably adhered cells.

We postulate two possible explanations for the ability of Arp2-deficient fibroblasts to spread, albeit at a slower rate. First, spreading can be mediated by two independent actin networks: 1) the lamellipodial network, or 2) the lamellar network. Live cell actin speckle microscopy shows that these two networks exist in an overlapping pattern of several cell types, with the lamellipodial network having significantly higher kinetics of assembly and disassembly [Iwasa and Mullins 2007; Ponti *et al.* 2004]. The lamellipodial network is linked to proteins involved in the dendritic nucleation model of leading edge assembly, among which are Arp2/3 complex, capping protein, and ADF/cofilin [Nicholson-Dykstra *et al.* 2005; Pollard 2007]. In *Drosophila* S2 cells, suppression of ADF/cofilin causes a lag in spreading rate and efficiency, but causes an increase in lamellipodium, while suppression of capping protein decreases the width of the lamellipodial network but has no effect on the lamellum [Iwasa and Mullins 2007]. In contrast, the lamellar network may be associated with proteins such as formins and tropomyosins. In *Drosophila* S2 cells, suppression of tropomyosin expands the lamellipodium and shrinks the lamellum [Iwasa & Mullins 2007]. Work by Gupton *et al.* 2005 demonstrated that Arp-inhibited PtK1 epithelial cells were able to migrate efficiently, but quantitative speckle microscopy revealed that the predominant

actin network in these cells was lamellar [Ponti *et al.* 2004]. Thus, the ability of Arp2-depleted 3T3 cells adopt an apparently normal morphology after extended times could suggest that the lamellar network is able to compensate for lack of the main actin nucleator in the lamellipodial network [Giannone *et al.* 2007; Gupton *et al.* 2007].

A second possible explanation is that the Arp2/3 complex concentration in Swiss 3T3 cell cytoplasm is in vast excess, such that residual Arp2/3 complex after knockdown is sufficient for assembly of limited lamellipodial protrusions, leading to slow cell spreading. Arp2/3 complex concentration is $> 1 \mu\text{M}$ in amoeba and in human neutrophils [Higgs *et al.* 1999], and we measure cytoplasmic concentration in Swiss 3T3 cells to be $1.5 \mu\text{M}$. Even 95% suppression efficiency would leave approximately 76 nM Arp2/3 complex, which may be sufficient to maintain some degree of spreading efficiency. The small spreading regions that occur on the sides of the linear protrusions in our Arp2-depleted 3T3 cells (Fig. 4B, D) and in Arp3-depleted Jurkats (Gomez *et al.* 2007, Movie S3) suggest residual Arp2/3 complex activity. The existing models of Arp2/3 complex-mediated dendritic nucleation assume non-limiting Arp2/3 complex concentrations [Mogilner and Edelstein-Keshet 2002]. It would be interesting to examine the threshold Arp2/3 complex concentration necessary to maintain dendritic nucleation in this model. Guillou *et al.* [2008] reported similar sheet-like protrusion from linear protrusions, an integrin- and Rac1-dependent process they termed “filopodia swelling”. Given the upstream role of Rac in Arp2/3 complex activation, this result supports the idea that the sheet-like protrusions present in our Arp2-depleted cells are due to residual Arp2/3 complex activity. Interestingly, the authors postulate that filopodia actually exist as precursors for lamellipodia.

Arp2/3 Complex depletion in Jurkats

Work by Billadeau and colleagues indicate that the immune synapse of activated lymphocytes is Arp2/3 complex dependent [Gomez *et al.* 2007]. Specifically, Jurkats depleted of Arp2 or Arp3 and stimulated to spread on anti-CD3 yielded a stellate morphology that strongly resembles our Arp2-depleted Jurkats on anti-CD3. Interestingly, live cell imaging of Arp3-depleted cells revealed remnants of lamellipodia-like protrusion between the stellate filopodia spikes. The authors interpreted this result to indicate that filopodia might actually “concomitantly direct Arp2/3 complex lamellipod formation” [Gomez *et al.* 2007]. Such filopodia-directed lamellipodial extension is reminiscent of the limited lamellipodial protrusion we observe in spreading Arp2-depleted fibroblasts. Analysis of the Arp2/3 complex depletion-induced “filopodia” in Jurkats revealed that these structures were not VASP- or EVL-dependent structures, nor were they dependent on two formins FMNL1 (FRL1) and DIA1 (mDia1) [Gomez *et al.* 2007]. It is possible that the Arp2/3 complex-depleted linear structures in both Jurkats and 3T3s could actually be an underlying lamellar network, or some other scaffold supporting lamellipodial protrusion.

Limitations of knockdown studies

An important consideration in evaluating effects of protein suppression studies is the actual total and residual concentration of the targeted protein. As mentioned above, mammalian cells contain micromolar quantities of Arp2/3 complex, which might be in vast excess over that needed for limited spreading. Even with loss of 90–95% of the total protein via siRNA or shRNA, we expect that 100 nM or more Arp2/3 complex may still be present. This quantity might be sufficient for general cell function and basal activity. If Arp2/3 complex is present in excess, observation of knockdown effects might only be observable 1) under extremely strong suppression, when the levels of Arp2/3 complex are below the threshold for basal function; or 2) when a cell is challenged to function beyond basal activity, for example, during replating. This variance in phenotypic penetrance due to both the extent of

protein suppression and extent of cellular activity may explain some of the differences in results observed amongst Arp 2/3 complex depletion studies.

Although other groups have successfully isolated stable Arp2/3 complex knockdown clones [Di Nardo *et al.* 2005], our attempts at isolating both fibroblasts and lymphocytes with constitutive Arp2 knockdown were unsuccessful. Similarly, Weber and colleagues [Harborth *et al.* 2001] found that the Arp2/3 complex may be essential for viability in HeLa cells. Whether this effect is due to Arp2/3 Complex activity in spreading and migration or in another as yet unidentified activity is yet to be determined.

Effects of Arp2-depletion on microvilli

The Convergent Elongation model was proposed to describe assembly of filopodia from Arp2/3 complex-nucleated branched filament networks [Svitkina *et al.* 2003]. In the model, a tip complex protects the barbed ends of filaments from capping to allow elongation of longer filaments after initial Arp2/3 Complex nucleation. Additionally, elongated filaments are bundled together by fascin or other proteins to form a thick linear protrusion. More recently, several groups have demonstrated that filopodia can be assembled via Arp2/3 Complex-independent mechanisms, such as through formins, suggesting that there might be multiple ways to achieve a linear protrusion [Pellegrin and Mellor 2005; Peng *et al.* 2003; Sarmiento *et al.* 2008; Steffen *et al.* 2006].

We previously reported lymphocytes lacking the Arp2/3 complex activator, Wiskott Aldrich Syndrome Protein (WASp) from both primary and cultured lymphocytes exhibit no defect in surface microvilli, suggesting that Arp2/3 complex does not play a role in the assembly or maintenance of these linear protrusions [Majstorovich *et al.* 2004]. Here, we demonstrate that Arp2-depletion has no effect on assembly of either the highly dynamic constitutively-exhibited surface microvilli of murine 300.19 pre-B lymphoma cells or the mitotic microvilli of Jurkat cells. Future studies will test whether these structures assemble by alternate mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Arp2 suppression in 3T3 Fibroblasts

(A) Western blot of Arp2 (upper) and actin (lower) levels in Control and Arp2-knockdown whole cell extracts. (B) Phase and immunofluorescent images depicting Control (upper) and Arp2-KD (lower) cell stained for Arp2 to demonstrate depletion of endogenous Arp2 in GFP + anti-Arp2-shRNA transfected cells. (C–D) Phase (left) and fluorescent images of polymerized actin (center) and GFP (right) in Control (C) and Arp2-KD (D) cells 78 hours post-transfection. Cells were plated onto glass coverslips immediately after transfection. (E–F) Phase contrast images of Control (E) and Arp2-KD (F) cells plated onto glass coverslips directly after transfection, then observed 78 hours post transfection by live cell imaging to monitor peripheral protrusion dynamics. (G–H) Enlarged phase contrast time lapse image of regions from Control (G) and Arp2-KD (H) depicted by boxes in D,E. Time points indicate time in minutes during time course. Bar: B,C = 20 μ m; D–G = 10 μ m.

Figure 2. Fibroblast Spreading Assay

SEM (A–C) and immunofluorescent (D–F) staining of 3T3s replated after trypsinization on PLL-coated coverslips and fixed after 20 min (A, D), 60 min (B, E) or 24 hours (C, F). SEM images taken at 500x, bar = 50μm. D–F) Phase (left), polymerized actin (middle) and tubulin (right) staining. Polymerized actin labeled with rhodamine-phalloidin. Image levels adjusted independently to reflect localization and organization of polymerized actin and tubulin. (G,H) Fluorescent time-lapse images of 3T3s expressing 6His-GFP-Actin (G) or GFP-Arp3 (H) during replating time course on PLL-coated coverslips. Bar: D–F = 20 μm, G,H = 10 μm.

Figure 3. Spreading time course of Arp2-knockdown 3T3 fibroblasts on PLL

Images of phase and fluorescently stained F-Actin and tubulin in Empty Vector Control (A) and Arp2-knockdown cells after 20 min, 60 min, 120 min and 8 hours after trypsinization and replating on PLL-coated coverslips. Fluorescent levels separately adjusted to optimize view of F-actin and tubulin in each image. Bar: A–B = 10 μ m. (C) Quantification of morphology of Empty Vector Control and Arp2-knockdown cells after 20 minutes of spreading on PLL-coated coverslips. (D) Quantification of number of Empty Vector Control and Arp2-knockdown cells adhered to coverslip and exhibiting a spread morphology similar to untransfected control cells (see Fig. 1).

Figure 4. Arp2-knockdown inhibits cell spreading and lamellipodial protrusion

(A–I) Phase contrast time lapse images of cells spreading on PLL-coated coverslips. (A) Untransfected control (A) and Arp2-knockdown (B) cells observed from 30–46 minutes after initiation of spreading assay. (C–E) Enlarged inset of peripheral lamellipodial protrusions (C) of control cell outlined in (A) and of lamellipodia-like protrusion along the length of pre-existing filopodia-like protrusions of Arp2-knockdown cells (D, solid box; E, hashed box) outlined in (B). Empty Vector Control (F) and Arp2-knockdown (G) cells observed 30–38 minutes after initiation of spreading assay. Enlarged inset of peripheral lamellipodial protrusions of control cell (H) outlined in (F) and of remaining lamellipodial protrusions of Arp2-knockdown cell (I) outlined in (G). Bar: A,B = 10 μ m; C–E = 5 μ m; F,G = 10 μ m; H,I = 2 μ m.

Figure 5. Arp2-depletion inhibits Jurkat ruffle formation

SEM (A) and fluorescent images of actin-rich ruffles (B) on the surface of Jurkat cells in suspension. SEM images of Control (C) and Arp2-KD (D) cells fixed in suspension 76–78 hours post-transfection. Bars: A–E = 2 μ m. Cells were evaluated for the presence of ruffles, microvilli, blebs and bald surfaces (E).

Figure 6. The microvilli of Arp2-depleted Jurkats are not a result of mitotic arrest
 SEM (A) and fluorescent (B) images of untransfected Jurkat cells demonstrating presence of occasional microvillar-covered cells, clearly seen by low-magnification SEM images (A) and by imaging the apical surface of cells stained for polymerized actin (B). Microvilli-covered cells are outlined by boxes. (C,D) SEM (upper) and immunofluorescent images (lower) of Untreated Control cells (C) and Nocodazole-blocked cells 45 minutes post-washout (D). Fluorescent images include rhodamine-phalloidin stained actin on dorsal surface (upper), anti-tubulin stained microtubules (middle) and DAPI-stained DNA (lower). (E) Quantification of surface morphology (by SEM analysis) of untreated and post-nocodazole washout cells. Cells were evaluated for presence of ruffles, microvilli, blebs or bald surfaces. (F) Mitotic Index of control and 45 min post-nocodazole washout cells. Immunofluorescent images were evaluated for total number of interphase and mitotic cells in general population. (G) Mitotic Index of Control and Arp2-depleted cells 76 hours after transfection. Quantification presented in F,G is from a single experiment that is representative of multiple nocodazole block and release trials.

Figure 7. Arp2-depletion has no effect on microvillar formation in 300.19 cells

Western blot analysis of Control and Arp2-KD 300.19 cells (A) stained with anti-Arp2. (B,C) Phase (left) and fluorescent images of the actin-rich puncta and the peripheral band of actin of GFP+ Control (B) and Arp2-KD (C) cells. SEM images of Control (D) and Arp2-KD (E) cells fixed in suspension. Bar: B–E = 5 μ m.

Table I
Comparison of Protrusion Dynamics

Protrusion rates of sheet-like protrusions were measured through frame-by-frame analysis of regions of cells that were actively extending. For replating assays, at least 5 cells were analyzed over the course of 10 minutes with at least 10 measurements taken per cell per time point. For protrusion of cells plated for extended times, at least 8 cells were measured over the course of 5 minutes, with at least 10 measurements taken per cell per time point. Measurements expressed in microns/minute.

	Sample	Protrusion Rate of Sheet-Like Protrusions	Difference from Control
3T3 Cells Spreading on PLL	GFP-Actin	2.77±1.50 µm/min	-
	Control	1.65±0.35 µm/min	-
	Arp2-KD	0.53±0.05 µm/min	68% decrease
3T3 Cells Plated for Extended Times	ON Control	3.60 ±0.58 µm/min	-
	ON Arp2KD	1.97±0.90 µm/min	45% decrease