

## Role of the actin cytoskeleton in acinar cell protein secretion

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### Abstract

All eukaryotic cells, including the pancreatic acinar cell, tightly control organization of their cytoplasm which in large part relies on the cytoskeleton. The cytoskeleton is comprised of three filament types: microtubules (tubulin), intermediate filaments (keratins), and microfilaments (actin). In addition to characteristics common to all cells, the pancreatic acinar cell is a polarized epithelial cell with a vectorial arrangement of the secretory machinery and apically-directed protein secretion, with important roles of the cytoskeleton in this polarity. The most investigated of the cytoskeleton elements in the acinar cell are actin-based microfilaments which are involved in regulated protein secretion. The development of the fluorescent Lifeact probe for realtime visualization of actin dynamics in live cells makes this an exciting time for the field. The dynamic rearrangement of the actin cytoskeleton associated with protein secretion is the focus of this review.

### Introduction

The actin cytoskeleton that is ubiquitous in nonmuscle cells is largely made up of  $\beta$ -actin and  $\gamma$ -actin, the products of the *ACTB* and *ACTG1* genes, respectively (2). These two actins differ by only 4 amino acids but they appear to have

different functions, as mutations in the two genes have very different effects (2). In the mouse acinar cell, *Actb* mRNA is expressed more than 2-fold greater than *Actg1* (**Table 1**); presumably their protein levels match this expression although this has not been measured in the pancreas. In gastric parietal cells,  $\beta$ -actin is more localized to the apical cortical cytoskeleton whereas  $\gamma$ -actin is more localized along the basolateral plasma membrane (BLPM) (48). If the same is true in the acinar cell, then  $\beta$ -actin is more likely to be important in protein secretion which is also localized to the apical surface of the cell. In the acinar cell,  $\gamma$ -actin may be involved in the BLPM blebs that form under supraphysiologic stimulation. From here on, actin will be referred to as if it were a single gene/protein.

Results from studies of the actin cytoskeleton in the exocrine pancreas are the primary source for this review. However, there are many gaps in our knowledge regarding exocrine pancreas function. When data from pancreas are lacking, results from other cell types that can reasonably be assumed to be true of the acinar cell are briefly discussed. It should be noted that even though there are commonalities shared by all cells with a regulated secretory pathway [e.g., regulation by small GTPases and use of SNAREs to mediate granule/vesicle - plasma membrane (PM) recognition and fusion (19)], there can be important differences.

**Table 1** Microarray analysis of mouse pancreas for actin and associated proteins

<b>Gene</b>	<b>Protein</b>	<b>Value*</b>	<b>Function / Notes</b>
<i>Actb</i>	Actin, beta, nonmuscle	20407	Actin
<i>Actg1</i>	Actin, gamma 1, nonmuscle	8659	Actin
<i>Actg2</i>	Actin, gamma 2, smooth muscle	1357	Actin
<i>Actn1</i>	Actinin, alpha 1	466	MF cross-linker
<i>Actn4</i>	Actinin, alpha 4	608	MF cross-linker
<i>Actr2</i>	ARP2 actin-related protein 2	2529	Branched MF nucleation on existing MF
<i>Actr3</i>	ARP3 actin-related protein 3	2952	Branched MF nucleation on existing MF
<i>Arpc2</i>	Actin related protein 2/3 complex, subunit 2	1745	Branched MF nucleation on existing MF
<i>Arpc3</i>	Actin related protein 2/3 complex, subunit 3	1178	Branched MF nucleation on existing MF
<i>Brk1</i>	Brick1	2966	SCAR/WAVE actin-nucleating complex
<i>Capzb</i>	Capping protein (actin filament) muscle Z-line, beta	1517	Caps barbed end of MF, inhibits elongation
<i>Cfl1</i>	Cofilin 1	2089	Depolymerizes MF
<i>Diap1</i>	Diaphanous homolog 1	1500	Formin family; Rho effector; MF nucleation and elongation
<i>Dstn</i>	Destrin	3654	Depolymerizes MF
<i>Ezr</i>	Ezrin	552	MV protein; links PM to MF
<i>Flii</i>	Flightless 1 homolog	2998	Gelsolin-like, MF severing
<i>Flnb</i>	Filamin, beta	650	Actin cross-linker; links PM to cytoskeleton
<i>Fscn1</i>	Fascin	4438	Actin bundling
<i>Gsn</i>	Gelsolin	2960	MF severing
<i>Mprip</i>	Myosin phosphatase Rho interacting protein	2893	Interacts with actin and myosin; function not clear
<i>Mylk</i>	Myosin light chain kinase	861	Activates myosin
<i>Rac1</i>	Rac1	4918	Small GTPase; WASP/WAVE, APR2/3 are effectors
<i>RhoA</i>	RhoA	1372	Small GTPase; Diap1 and ROCK1 are effectors
<i>Rock1</i>	Rho-associated coiled-coil containing protein kinase 1	378	RhoA effector; regulates actin cytoskeleton
<i>Shroom3</i>	Shroom3	1204	ROCK1 activation; MF assembly
<i>Sptbn1</i>	Spectrin beta, nonerythrocytic 1	2808	Actin cross-linker; links PM to cytoskeleton
<i>Svil1</i>	Supervillin	3161	Links PM to cytoskeleton
<i>Tln1</i>	Talin 1	1273	Links integrins to actin cytoskeleton
<i>Tmod3</i>	Tropomodulin 3	2187	Caps pointed end of MF, inhibits disassembly
<i>Vcl</i>	Vinculin	312	Links integrins to actin cytoskeleton

From microarray analysis of mouse pancreas (<http://www.ncbi.nlm.nih.gov/geo/>, accession GDS567). Data are means from 3 wild type young adult mice. See (18) for details. \* Value: "normalized signal count data, reflecting the relative measure of abundance of each transcript" as defined on the GEO website. This list is not comprehensive. Many actin cytoskeletal genes were expressed below detection limits, or were not represented on the microarrays.

For example, the role of actin coating of granules/vesicles during the content release process may serve different functions related to the speed of product release required (see [4 E. Dynamics of acinar actin in the stimulated state](#) below).

## Actin Basics

Actin is a 42 kDa protein that exists in two distinct forms in the cell: monomeric (globular or G-actin) ; and polymeric (filamentous or F-actin). Actin monomers have the inherent behavior of self-assembly into filaments and prevention of inappropriate polymerization is controlled by several G-actin binding proteins that stoichiometrically outnumber G-actin by more than 3-fold (47). However, in general the majority of a cell's actin is in the form of F-actin, estimated to comprise ~75% of the total (47). The structure of F-actin microfilaments (MF) is a polarized double helix such that the barbed (+) end is where new monomers are primarily added and the pointed (-) end is where F-actin primarily releases actin monomers. Actin is an ATPase, and for G-actin the ATP-bound form predominates and is thus primed for addition to an actin filament. Once added to the filament, the ATPase is activated and ATP is hydrolyzed to ADP.

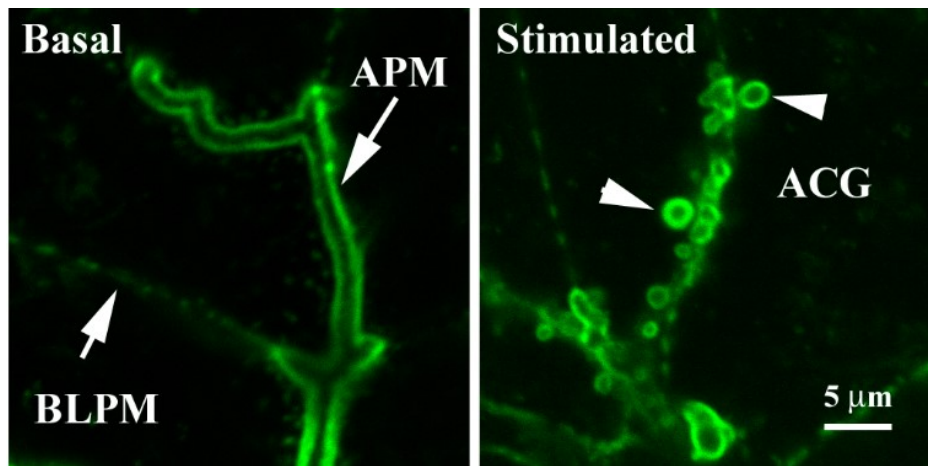
F-actin is highly dynamic and there are numerous proteins that regulate the assembly and disassembly of MF. Interrogation of a published microarray study done in the author's lab(18) for actin cytoskeleton elements was performed and is presented in **Table 1**. This yielded a number of genes, the most highly expressed of which not surprisingly were  $\beta$ - and  $\gamma$ -actins, as well as several proteins known to bind to or regulate dynamics of actin. Many of these proteins directly interact with actin and are thus called actin binding proteins (ABP) (42). The majority of genes on this list and their functions were discovered using other cell systems and have not been investigated in the pancreatic acinar cell.

Microfilament (MF) structures in cells may appear to be highly stable structures, but they are constantly undergoing growth at the (+) end and disassembly at the (-) end, a process called treadmilling (38). Thus, the apparent stability of MFs is actually due to a dynamic equilibrium. It has been estimated that the ~1  $\mu\text{m}$  long MFs of the intestinal microvillus turns over every 20 min by treadmilling, and a similar rate is also believed to be true of the terminal web (TW) that underlies the apical plasma membrane (APM) (9). In addition to proteins that induce disassembly of MFs, there are numerous actin-binding proteins that regulate nucleation of new fibers and growth of existing fibers. Actin can exist as higher order bundles of multiple MFs in linear arrays as well as branched MF structures, with accessory proteins providing lateral cross-links (42).

## Actin Localization and Dynamics in the Acinar Cell

### Methods to Visualize F-actin

Most studies of actin in the acinar cell have been of MFs and usually after chemical fixation of the cells in either the unstimulated basal state, with a secretory stimulus, and/or after a specific experimental manipulation of the cells. The most commonly used technique is fluorescently tagged phalloidin, a fungal toxin with a high affinity for F-actin (see [Pancreapedia](#) articles and images therein: [Rab3](#); [Visualization of exocytosis in pancreatic acinar cells by fluorescence microscopy](#); [Rab27](#)). An example of FITC-phalloidin labeling of mouse acini from the author's lab is shown in **Figure 1**, which shows the MFs of acinar cells under basal and stimulated conditions. Phalloidin is a stabilizer of F-actin MFs so when used in living cells it perturbs the actin cytoskeleton. This property limits use of phalloidin to static imaging of F-actin after fixation, and precludes its use to visualize dynamics of actin in live cells where other techniques are used (see below).



**Figure 1. FITC-phalloidin staining of F-actin in mouse acinar cells under basal and stimulated conditions.** Mouse acini were incubated without (Basal) or with 1  $\mu\text{M}$  carbachol (Stimulated). The cells were fixed in suspension with 2% formaldehyde, 0.1% saponin to permeabilize cell membranes, and 1 mM  $\text{MgCl}_2$  and 5 mM EGTA to stabilize F-actin. The cells were then stained with 0.6  $\mu\text{M}$  FITC-phalloidin and imaged on a confocal microscope, collecting 0.5  $\mu\text{m}$  optical sections. These images are single optical slices. The acinar lumen is bounded by the APM (left panel), under which is the strongly labeled TW. The BLPM (left panel) is much weaker stained than the APM. After stimulus, there are actin coated granules (ACG) along the APM (right panel, arrowheads) and the TW is more weakly stained. [These images were preliminary data for the work presented in (37).]

F-actin can also be recognized in standard transmission electron microscopy as electron dense  $\sim 6$  nm diameter filaments (smaller than microtubules,  $\sim 24$  nm; and intermediate filaments,  $\sim 10$  nm).

Until recently, MFs have been difficult to visualize in living cells without perturbing the structure and function of the cytoskeleton. Adding tags such as green fluorescent protein (GFP) to  $\beta$ -actin perturbs the actin cytoskeleton into which it is incorporated [discussed in (14)]. A newer approach is to make chimeric fluorescent proteins from ABPs. Using such an approach the Lifeact reporter was made, which consists of a 17 amino acid actin binding domain from the ABP140 protein, fused to GFP (31). This chimeric protein has a 30-fold greater affinity for F-actin than G-actin, was found to not interfere with known activities of the actin cytoskeleton, and it faithfully reproduces known F-actin staining patterns in a variety of cell types even under dynamic situations where the actin cytoskeleton is being remodeled (14). This

construct was later used to make a transgenic mouse expressing the fluorescent F-actin reporter in all tissues, called the Lifeact mouse (32).

G-actin can also be visualized using fluorescently tagged DNase I, which binds to both G- and F-actin, with higher affinity to the former (21), although an analysis of DNase I inhibition by highly purified G- and F-actin concluded that both forms have similar affinity for DNase I (25). An example of fluorescent DNase I staining of G-actin in the acinar cell shows a diffuse cytoplasmic distribution which is not very informative (27). Visualization of G-actin has not been used to study the actin cytoskeleton of the acinar cell to any extent. Nor has there been systematic investigation of G-actin ABPs in the acinar cell.

#### **Acinar Cell Actin in the Resting State**

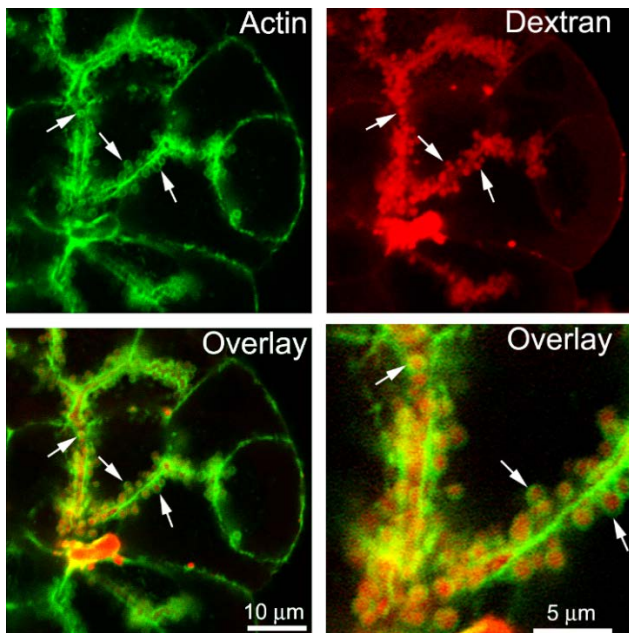
The single largest pool of F-actin in the acinar cell is in the TW (4), also called subapical actin or cortical actin (**Fig.1, left panel**).

**Table 2** Drugs and reagents that affect the actin cytoskeleton and their effects on pancreatic acinar cell cytoskeleton and amylase release

Agent	Mechanism	Cytoskeleton Effects	Amylase Release / Other	Ref
LatA	Binds G-actin monomers, inhibiting addition to MF	no effect on ACG; loss of actin cables		15
		TW resistant; BPLM MFs lost	inhibits / 28% decrease in MF in 10min; 44% decrease at 2hr	7
			inhibits / prevents BLPM blebs by supramaximal CCK	6
		loss of ACG	/ done at room temperature	27
LatB	Binds G-actin monomers, inhibiting addition to MF		no effect /	43
		loss of ACG; reduces TW	/ early pore closure	20
		loss of ACG	no effect /	17
		loss of TW	/ perturbs Ca <sup>2+</sup> -signaling	36
Cytochalasin	Binds to barbed end of MF; disassembly of existing MFs		no effect /	43
		reduces lifetime of ACG		27
		loss of TW	reduces / dilation of lumen	45
		loss of TW	reduces; rebounds after washout / dilates acinar lumen with stimulation; compound exocytosis increases	44
Jasplakinolide	Stabilizes existing MFs, enhances MF nucleation		reduces; prevents high-dose CCK inhibition / causes BLPM blebs in absence of stimulation	6
BDM	Inhibits myosin ATPase		totally blocks / prevents loss of MF by latrunculin or cytochalasin	40
BDM & LatB		ACG accumulate		40
BDM & Cyto		no effect on ACG		40
C3 (Rho-toxin)	ADP-ribosylates & inactivates Rho		inhibits /	7
		loss of ACG		27
			/ partially blocks supramaximal CCK inhibition of amylase release	6
CA-Rho	Stimulates downstream effectors	increases total F-actin	enhances / prevents supramaximal CCK inhibition of amylase release; causes BLPM blebs	6
CA-Rac	Stimulates downstream effectors	increases total F-actin	enhances / prevents supramaximal CCK inhibition of amylase release; causes BLPM blebs	6
DN-Rho	Inhibits downstream effectors		reduces, both phases affected /	6
DN-Rac	Inhibits downstream effectors		reduces, primarily late phase / partially blocks supramaximal CCK inhibition of amylase release	6
DN-Rho & DN-Rac	Inhibits downstream effectors		reduces, slightly greater than sum of individual DN effects /	6
CA-mDia	Recruits G-actin for MF growth	no effect on ACG		15
DN-mDia	Blocks mDia-induced MF growth		no effect /	15
β -thymosin	Binds G-actin monomers, inhibiting addition to MF	no effect on TW		26
Gelsolin fragment	Binds G-actin monomers, inhibiting addition to MF	no effect on TW		26
Phalloidin	Stabilizes existing MF		inhibits /	26

Empty fields indicate data was not reported for that parameter. Abbreviations: ACG: actin-coated granules; BDM: 2,3-butadione monoxime; CA: constitutively active; Cyto: cytochalasin; DN: dominant negative; Lat: latrunculin; TW: terminal web





**Figure 2. ACG are open to the lumen and accumulate fluorescent dextran from the medium.** Acini were stimulated in the presence of fluorescent lysine-fixable dextran (red) and then fixed and costained with FITC-phalloidin, and imaged on a confocal microscope. The enlarged image (lower right) clearly shows that ACG also have fluorescent dextran in their lumina, demonstrating access of the ZG lumen to the medium during secretory stimulation. [Courtesy of John A. Williams, Unpublished]

The TW is a dense arrangement of MF that forms a continuous sheet-like network ~200 nm thick (13) under the APM. Among ABPs, members of the red blood cell spectrin family cross-link TW MFs and also link the TW to perpendicular MF that extend into the microvilli (MV), as shown in intestinal epithelium (16). As compared to the villus epithelium of the small intestine where MV are best characterized, the acinar cell MV are relatively sparse. A second actin-based structure is a ring of MFs that are attached to the adherens junctions near the apical surface and encircle each acinar cell. Because this is a ring-shaped structure it is less easily visualized than the sheet-like TW. There are also recently-described MF bundles that project perpendicularly from the TW into the cytoplasm that are called actin cables (15). Lesser amounts of MFs are found along the BLPM of the acinar cell (**Figure 2, left panel**).

Because of the large amount of F-actin in the TW, it can be difficult to visualize the other sites of MFs when using fluorescent labeling approaches. For example, the recently described actin cables that run perpendicular to the TW in acinar cells are not observed using phalloidin staining, but are visualized using a chimeric ABP domain fused to GFP (Lifeact, see below) (15).

### **Effects on Secretion of Pharmacological Agents and Molecular Approaches that Perturb the Actin Cytoskeleton**

There is a large data set about the acinar cell actin cytoskeleton obtained using a variety of approaches. Because the actin cytoskeleton is so important to eukaryotic cells, there are numerous naturally occurring substances produced by a variety of organisms that interfere with the organization of actin, and many of these are available as research tools. Also, investigators have produced constitutively active (CA) and dominant negative (DN) constructs to stimulate or inhibit upstream pathways that control the actin cytoskeleton. A summary of these toxins and reagents and their effects on the actin cytoskeleton and acinar cell amylase release is given in **Table 2**. Some of the reported effects of a particular agent are contradictory but these are likely attributable to differences in concentration of the agent (e.g. latrunculins have been used over the wide range of 0.3  $\mu$ M to 100  $\mu$ M), time of exposure, and temperature at which the experiment was performed [discussed in (7)]. The importance of a specific agent's concentration is illustrated by the finding discussed below that a small perturbation of the actin cytoskeleton increases amylase release, whereas stronger perturbation blocks amylase release (26). The data covered here attempts to give the consensus of the available data.

### **Signals that Affect the Actin Cytoskeleton**

The principal mechanism of acinar cell stimulation begins with ligand binding to membrane receptors coupled to G proteins that feed into several pathways. With respect to the acinar cell actin

cytoskeleton, the known pathways are activation of the small GTPases RhoA (via  $G\alpha_{13}$ ) and Rac1 (via  $G\alpha_{13}$  and  $G\alpha_q$ ) (35). These activated small GTPases in turn activate their downstream effectors such as Diap1 and ROCK1; and WASP/WAVE and ARP2/3, respectively, that lead to actin cytoskeleton remodeling.

As discussed below, a major downstream effector of Rho activation is the formin family member Dia. In *Drosophila*, the formin Diaphanous is activated by the small GTPase Rho, resulting in nucleation and formation of linear F-actin filaments that comprise the TW of epithelial cells (23). Although not yet experimentally verified, Rho activation in the mammalian pancreas is expected to activate mDia1 and it has been shown in mouse acinar cells that constitutively active (CA)-mDia1 leads to F-actin cables perpendicular to the apical PM (15). It was suggested these F-actin cables serve to recruit zymogen granules (ZG) through the TW to the apical membrane during secretory stimulation (15). Consistent with the requirement for apical MF in secretion, protein release into the tracheal lumen of *Drosophila* lacking Dia, which also lack TW F-actin, was absent. A notable difference between *Drosophila* and mammalian acinar cells is that Dia in *Drosophila* is required for formation of the TW of epithelia (23); whereas in mouse acini, the only observed role of mDia was in formation of the F-actin cables perpendicular to the APM, and the TW persisted in the presence of a DN-mDia construct (15). Also in contrast to *Drosophila*, mouse acini expressing dominant negative (DN)-mDia1 have unchanged basal and stimulated amylase release (15). Clearly, the roles of Dia/mDia1 differ in *Drosophila* and mouse pancreatic acini.

### **Dynamics of Acinar Actin in the Stimulated State**

Actin undergoes a variety of changes during secretory stimulation. The TW in the resting acinar cell is a barrier to zymogen granule (ZG) exocytosis, and the F-actin here must be rearranged to allow ZG access to the APM when

the cell is stimulated (26). Using streptolysin O-permeabilized acinar cells, it was demonstrated that mild perturbation of the actin cytoskeleton by adding low concentrations of G-actin sequestering proteins ( $\beta$ -thymosin or a gelsolin fragment; Table 2) resulted in amylase release, especially the early phase of release (26). Interestingly, even when  $Ca^{2+}$  was chelated at low levels using EGTA, mild perturbation of the actin cytoskeleton resulted in amylase release. If high concentrations of the G-actin ABPs were used that resulted in extensive loss of F-actin, there was reduced amylase release. Similarly, if the F-actin stabilizing drug phalloidin was introduced to permeabilized acini, amylase release was inhibited. Together, these data indicate that the TW is a barrier to ZG exocytosis and that, while some remodeling of the actin cytoskeleton is required for release, extensive disruption or stabilization of F-actin is detrimental to protein secretion.

The dynamics of G-actin per se have not been investigated in relation to acinar cell stimulation. However, there have been measurements of the relative changes in the total amount of F-actin in acinar cells, which show that an increase in polymerized actin is associated with amylase release (6). Furthermore, pharmacological reduction of F-actin (1.0  $\mu$ M latrunculin, a G-actin sequestering agent, Table 2) inhibits CCK-stimulated amylase release from mouse acini without affecting  $Ca^{2+}$  dynamics (7). Since an acute increase in F-actin means less G-actin, these data add to the concept that dynamic changes in actin are involved in protein secretion in the acinar cell.

A similar conclusion regarding actin remodeling during secretion can be drawn from experiments using supramaximal CCK which induces large alterations in the actin cytoskeleton and shows inhibition of amylase release (10, 40). When DN-Rho and DN-Rac (**Table 2**) constructs were used, they prevented supramaximal CCK inhibition of amylase release, and at the same time lessened

reorganization of the actin cytoskeleton (6). In that study a low dose of jasplakinolide (1  $\mu$ M) also prevented supramaximal CCK inhibition of amylase release, but a higher dose (3  $\mu$ M) inhibited amylase release at all concentrations of CCK, consistent with the effect of jasplakinolide reported by Valentijn et al (43) (**Table 2**). So again, these results show there is a 'sweet-spot' for amylase release with respect to organization of the acinar cell actin cytoskeleton.

Remarkably, during exocytosis the ZG membrane (ZGM) becomes 'coated' with F-actin (actin-coated granules, ACG) (43) (**Figure 1, right panel**). In the acinar cell, the role of the F-actin coat has been proposed to stabilize the fused ZG at the PM (20, 41). This may be related to the duration of acinar protein secretion (seconds, which is long relative to neuronal exocytosis which occurs on a millisecond time scale), which may be required for the highly concentrated digestive enzymes to solubilize and diffuse out of the granule lumen into the beginning of the pancreatic ductal system. In other cells with the regulated pathway whose contents are more rapidly solubilized, the F-actin coat appears to flatten the fused vesicle thus quickly forcing out the secretory product. The potential differences in the roles of F-actin granule/vesicle coats in various secretory cells are discussed in some excellent reviews (28, 29). Interestingly, the G-actin sequestering drug latrunculin blocks coating of ZG with actin but does not prevent ZGM-PM fusion (17).

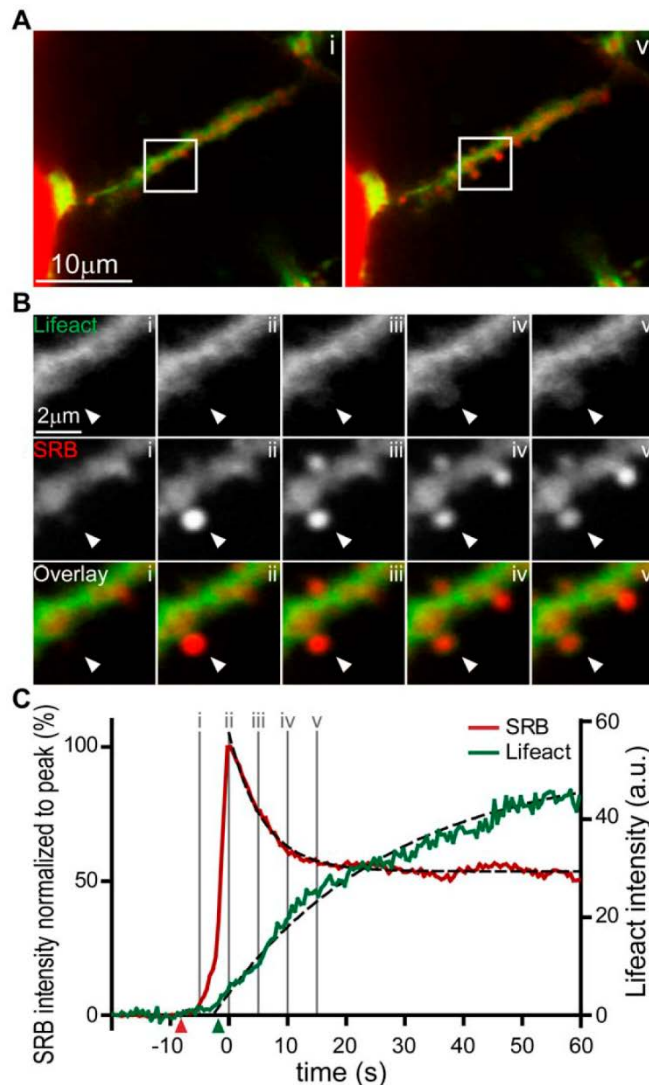
Using the C3 Rho toxin from *Clostridium botulinum*, it was shown that ACG formation is Rho-dependent (27). When acini were pretreated with the C3 toxin and then stimulated to secrete, ACG were not observed but large vacuoles formed that were filled with the fluorescent extracellular tracer, showing that granule fusion had still occurred but that the individual granules in the absence of actin coating appear to fuse into

a large vacuole. Thus, the rapid formation of ACG in stimulated cells appears to be Rho-dependent, and ACG formation may prevent vacuole formation. Amylase release was not measured in these experiments.

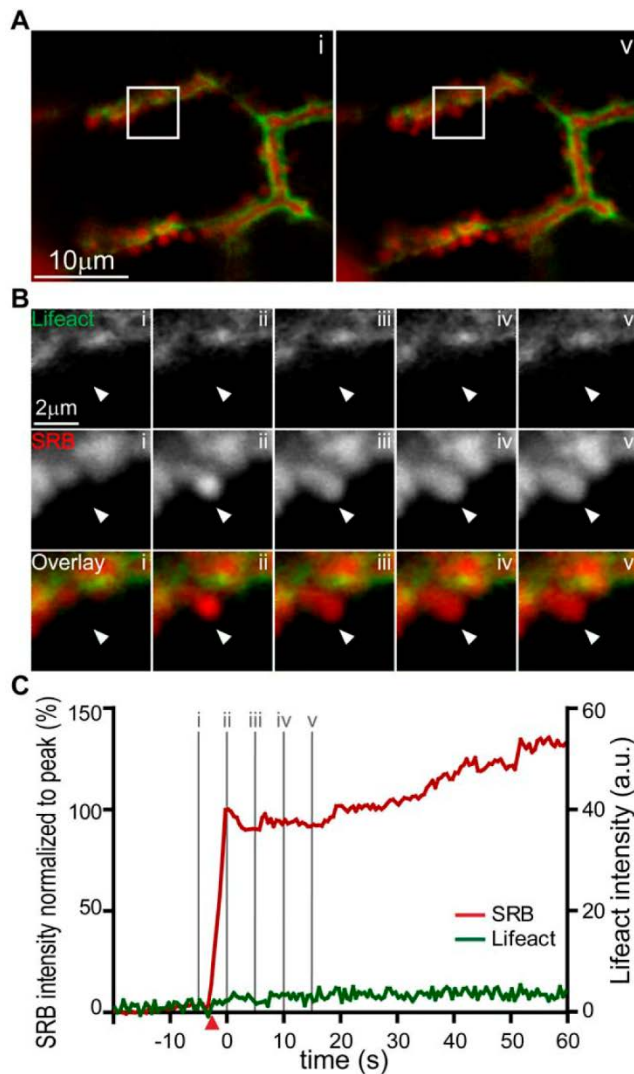
The timing of F-actin coating of ZG relative to ZGM-PM fusion has evolved since its discovery. Early descriptions of ACG suggested that the coat developed before ZGM-PM fusion, based on failure of entry of a fluorescent lectin (WGA) from the medium into ACG, indicating their lumens were not yet accessible to the medium (43). Later experiments concluded that the F-actin coat forms after membrane fusion, while the exocytic pore is still open (27, 41) (also see Pancreapedia article [Visualization of exocytosis in pancreatic acinar cells by fluorescence microscopy](#)). This conclusion was based on the observation that only ZG accessible to the acinar lumen (able to take up lysine-fixable fluorescent dextran from the medium) were actin-coated and no ZG without fluorescent dextran were actin-coated. An example of acini stimulated in the presence of a lysine-fixable dextran followed by fixation and fluorescent phalloidin staining is shown in **Figure 2**. This clearly shows dextran in the lumens of ACG. Subsequent work using a soluble tracer (sulforhodamine B) with living cells convincingly showed that access to the ZG lumen occurred on average 6.7 sec before appearance of the actin coat, as visualized in acini from a transgenic mouse expressing Lifeact-GFP (17) (**Figure 3**). This study also showed that latrunculin blocked formation of ACG while ZG fusion was not inhibited (**Figure 4**). Hence, ZGM-PM fusion occurs rapidly followed by actin coating of the fused ZG while it is still in the ' $\Omega$ ' shape, and that fusion per se is independent of ACG formation.

A different interpretation of the timing of ZG fusion and ACG formation has been made recently by Geron et al., using Lifeact-GFP, expressed in acini by adenoviral infection (15).





**Figure 3. Actin coating of ZG occurs shortly after ZGM-PM fusion.** [From (17), Fig.4] "Real-time imaging of exocytic fusion events and F-actin coating. (A) Low magnification images show a lumen lying diagonally between two acinar cells identified with SRB (red) and Lifact-EGFP (green) in the sub-apical region. (A, i) is an image taken before the appearance of exocytic events at the time point indicated "i" on the graph of fluorescence intensity over time in panel (C). (A, v) Is an image at a time point after induction of a number of exocytic events which can be seen as bright spots of SRB fluorescence along the lumen; the time point "v" is indicated on the lower graph (C). (B) Shows an image sequence from an enlarged region (box shown in A) of Lifact-EGFP and SRB and the overlay, for two exocytic events. The images were taken at the time points i, ii, iii, iv, and v as indicated on the graph in panel (C). (C) is a graph of fluorescence changes over time taken from a region of interest placed over the lower exocytic event (indicated by an arrowhead). SRB fluorescence is plotted normalized to the peak and rises rapidly to a peak and then decays to a plateau. The simultaneously recorded Lifact-EGFP signal, plotted in arbitrary fluorescence units, rises slowly and nearly reaches a maximum by the end of the record. The starting points of the SRB and Lifact-EGFP signals, as determined by a positive deflection of the signal by more than 5 times the standard deviation of the signal noise, are shown by the colour-coded triangles on the X axis. The black dotted lines were mono-exponential fits to the data with  $\tau$  values of 6.9 s (SRB) and 29.4 s (Lifact-EGFP). doi:10.1371/journal.pone.0039815.g004"



**Figure 4. Latrunculin A abolishes actin coating of ZG but does not inhibit ZGM-PM fusion. [From (17), Fig.5]** "Latrunculin treatment abolishes F-actin coating of fused granules. (A) Low magnification images show complex lumens, identified by SRB (red) and Lifeact-EGFP fluorescence (green), lying between the cells within a pancreatic fragment. (Ai) is an image taken before the appearance of exocytic events, (Av) is taken after, at the time points "i" and "v" as indicated on the graph in panel (C). (B) Shows an image sequence from an enlarged region (box shown in A) of Lifeact-EGFP and SRB and the overlay, for a single exocytic event. The images were taken at the time points i, ii, iii, iv, and v as indicated on the graph in panel (C). (C) Is a graph of fluorescence changes over time taken from a region of interest placed over the exocytic event (indicated by an arrow). SRB fluorescence is plotted normalized to the first, rapid peak and shows a rapid rise followed by a slower increase. The simultaneously recorded Lifeact-EGFP signal, plotted as arbitrary fluorescence units, shows only very small changes over time. doi:10.1371/journal.pone.0039815.g005"

This study was of the role of the formin family member diaphanous (DIAP1, called mDia in mouse), which is a Rho effector whose activation leads to linear MFs in the pancreatic acinar cell that they called actin cables. They state in the introduction to their paper that 'Secretory vesicles

undergo actin coating *before* fusing to the apical membrane...' (emphasis added) and they cite (27, 28, 43). One can observe in their videos accompanying the paper ACG moving apparently along F-actin cables that run perpendicular to the apical PM. Since these ACG appear deeper

inside the cells than the PM, it was concluded that coating preceded ZGM-PM fusion. Even though they had

included FM4-64, a fluorescent membrane tag that should enter fused ZG and label their membranes, in none of the data shown is FM4-64 fluorescence ever observed in ZG. Therefore, it is not clear that the experiments actually could reveal when ZGM-PM fusion occurred. A more consistent interpretation of the role of mDia-induced actin cables is that fused ZG are moving along these cables near the apical PM.

Another issue with their interpretation is that it does not concur with what was observed when the cables were interfered with. They showed that expression of a CA- mDia1 resulted in an increase in actin cables, and that a DN-mDia1 mutant caused a strong decrease in the number of cables. Neither of these affected basal or stimulated amylase release. If the cables are an important mechanism to move ZG in the cytoplasm to the PM so they can then fuse, one would expect a decrease in stimulated amylase release when the cables are decreased, which was not observed. The DN-mDia1, which reduced cable formation, caused an increase in ACG fused to one another, which was interpreted as compound exocytosis. So, it would appear that the role of these mDia1 induced actin cables is to keep ZG separated such that individual granule fusion predominates and minimizes compound exocytosis.

It is likely an issue in these disparate results regarding the timing of ACG formation whether the acinar preparation was optimal for allowing access from the medium to the acinar lumen. This is discussed in (20, 46), where it is shown that the vigor of the tissue dissociation technique can yield groups of cells ranging from multi-acinar clusters to single acini or even smaller than acinar-sized groups that retain normal acinar secretory function. To allow access of the bathing solution to the acinar lumen, groups consisting of as few

as 3-6 cells with a short unhindered connection between the acinar lumen and the medium is optimal. [In my lab, we could not get lysine-fixable dextran into fused ZG (37) as achieved by other labs, likely because my 'acini' were actually larger multi-acini clusters.]

Although mDia is a downstream effector of activated Rho, introduction of DN-mDia (15) and DN-Rho A (6) in acinar cells does not produce the same effects (**Table 2**). DN-mDia does not affect amylase release whereas DN-Rho inhibits amylase release. These different effects on amylase release indicate that mDia is not the sole target of activated Rho in the acinar cell, and perhaps another of the Rho effectors is involved (39).

The conclusion that the F-actin coat develops rapidly after ZGM-PM fusion contributes to the idea that this coat functions to stabilize the pore in the open state, providing sufficient time for the stored zymogens to become soluble and diffuse into the acinar lumen. An actin coat attached along the cytosolic surface of the fused ZG would stiffen this membrane, preventing closure of the energetically-unfavorable pore. The presence of the nonclassical myosin Vc (Myo5c) on ZG, possibly tethered to the membrane by the small GTPase Rab27B (11, 12) is believed to provide a motive force to move ZG along the TW. Another myosin, Myo2A, is localized to the apical pole of acinar cells, is phosphorylated when the cell is stimulated, and when its activity is inhibited access of the ZG lumen to the soluble markers in the medium is reduced (5). This was interpreted as more rapid fusion pore closure when this myosin activity is inhibited. Also, when the TW was disassembled using cytochalasin treatment, the acinar lumen enlarged under stimulation but amylase was not released; when the cytochalasin and secretory stimulus was washed out, the TW recovered and amylase was released from the already fused ZG (44). It was suggested that contractile forces on the fused ZGM by actin were needed to force the ZG content out. These results

can be reinterpreted in light of the newer data indicating that MFs are required to maintain an open pore for protein release.

In addition to providing sufficient time for zymogens to dissociate and diffuse into the lumen, the actin-coat of fused ZG has been proposed to help counteract the expected hydrostatic pressure of these dissolving contents from the fused ZG in salivary glands (22). Also, there is compelling evidence that the fused ZGM transports electrolytes and fluid from the acinar cytosol into the lumen during protein secretion (see review in the Pancreapedia by Frank Thévenod; [Channels and Transporters in Zymogen Granule Membranes and their Role in Granule Function: Recent Progress and a Critical Assessment](#)) which will add to hydrostatic pressure within the fused ZG lumen.

While it has been shown that mDia1 is involved in the formation of actin cables in the acinar cell, and is regulated by the small GTPase Rho, it remains to be determined what controls localized disassembly of the TW required to allow passage of ZG to the PM for exocytosis. The F-actin structure that coats granules is likely to consist of a network of short interconnected linear filaments, or alternatively, of branching actin filaments. This is because linear F-actin filaments <10  $\mu\text{m}$  in length are rigid rods (8) and such long linear filaments could not be accommodated on the highly curved surface of granules which are  $\sim 1 \mu\text{m}$  in diameter (34). Consistent with this idea, a subunit of the ARP2/3 complex and N-WASP, factors involved in nucleation of branches on existing MFs, were immunolocalized to the region of ACG in stimulated acini (15). Based on this, Rac1 may be involved, because Rac1 has been shown to regulate WASP/WAVE ABPs that control nucleation/elongation of branched MF in other systems (30). However, WASP nucleates a new branch on existing F-actin and proteomic analysis of highly purified ZGM revealed the presence of small GTPases and molecular motors (dynein, myosins) but not actin or other

cytoskeletal elements (11, 12, 33). On the other hand, ultrastructural immunogold localization showed actin in the region of ZG (1, 3). Together these results suggest that actin in the ZG region of the cell is either loosely bound or not bound at all to the ZGM. In any case, it appears F-actin is not present on ZG before fusion, so there needs to be a process to nucleate new MF on the fused ZGM and at this time, how this occurs remains to be identified.

### **After the Stimulus Ends**

Once the fused ZG has released its content and exocytosis can be considered complete, the actin cytoskeleton has the further role of helping drive compensatory endocytosis. In order to maintain the balance of membranes between apical and basolateral domains, the ZGM added to the apical surface during protein secretion must be taken back into the cell. The endocytic process is believed to retrieve the ZGM in a clathrin-mediated fashion, and this also requires the actin cytoskeleton (44). It was observed in cytochalasin D treated acinar cells that the APM became dilated and pits on this surface were immunoreactive for the clathrin adaptor AP-2, clathrin, dynamin, and caveolin, all of which are components of various endocytic mechanisms. When cytochalasin was washed out, these proteins rapidly disappeared from the APM and the luminal membrane recovered to its prestimulus size. From this it was suggested that the actin cytoskeleton is also involved in the compensatory endocytosis process that follows protein secretion. The roles of actin in endocytosis are well known (24).

### **Summary**

There are several filamentous actin structures with important roles in acinar cell protein secretion: the TW, ACG, and actin cables. There is also BLPM associated F-actin which is involved in membrane blebbing under supramaximal stimulation. The recent development of the fluorescent Lifeact probe has begun to reveal the

dynamics of the actin cytoskeleton with new insights into post-fusion actin coating of ZGs and the discovery of actin cables perpendicular to the TW. It is expected this tool will continue to be

utilized for greater in depth understanding of the actin cytoskeleton and its roles in protein secretion from the acinar cell.

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