ABSTRACT

From yeast to man, an evolutionary distance of 1.3 billion years, the F-actin filament structure has been conserved largely in line with the 94% sequence identity. The situation is entirely different in bacteria. In comparison to eukaryotic actins, the bacterial actin-like proteins (ALPs) show medium to low levels of sequence identity. This is extreme in the case of the ParM family of proteins, which often display less than 20% identity. ParMs are plasmid segregation proteins that form the polymerizing motors that propel pairs of plasmids to the extremities of a cell prior to cell division, ensuring faithful inheritance of the plasmid. Recently, exotic ParM filament structures have been elucidated that show ParM filament geometries are not limited to the standard polar pair of strands typified by actin. Four-stranded non-polar ParM filaments existing as open or closed nanotubules are found in Clostridium tetani and Bacillus thuringiensis, respectively. These diverse architectures indicate that the actin fold is capable of forming a large variety of filament morphologies, and that the conception of the “actin” filament has been heavily influenced by its conservation in eukaryotes. Here, we review the history of the structure determination of the eukaryotic actin filament to give a sense of context for the discovery of the new ParM filament structures. We describe the novel ParM geometries and predict that even more complex actin-like filaments may exist in bacteria. Finally, we compare the architectures of filaments arising from the actin and tubulin folds and conclude that the basic units possess similar properties that can each form a range of structures. Thus, the use of the actin fold in microfilaments and the tubulin fold for microtubules likely arose from a wider range of filament possibilities, but became entrenched as those architectures in early eukaryotes.

The history of the eukaryotic actin filament structure

Actin has fascinated scientists for 75 years. We begin by charting the history of the elucidation of the eukaryotic actin filament structure. Actin, in its filamentous form (F-actin), was first discovered by Straub in the early 1940s as an integral component of muscle. Twenty years later, Hanson & Lowy used the novel structural tool of the early 1960s - electron microscopy – to show that F-actin, when polymerized in vitro, forms straight right-handed helices composed of 2 tightly intertwining strands. Using myosin, which binds F-actin strongly in the absence of nucleotide (corresponding to the rigor state of muscle), Hugh Huxley, who invented negative stain and spearheaded the early stages of structural electron microscopy, observed a unique ‘arrow head’ pattern under the electron microscope. He concluded that the 2 strands forming the F-actin helical filament were in the same orientation and the filament therefore is polar. Huxley’s myosin labeling technique for F-actin was exploited several years later to show that not only muscle cells but eukaryotic cells contain substantial amounts of F-actin, and Pollard and colleagues in 1975 determined that the “barbed” and “pointed” ends of the filament, indicated by the arrow heads, related to the fast and slow growing ends of the filament.

Revolutionary work by Toshio Yanagida in the mid-1980s showed that actin filaments could be visualized with fluorescence microscopy by labeling with rhodamine-phalloidin, which binds tightly at the interface between 3 actin protomers. This methodology aided the characterization of a myriad of cellular proteins...
associated with actin, many of which regulate the ability of actin to polymerize or depolymerize.\textsuperscript{26} In 1990, back-to-back publications of the actin monomer X-ray structure\textsuperscript{9} with the determination of its orientation within the filament, based on fitting of the monomer structure into X-ray fiber diffraction data, produced the “Holmes” model of the filament.\textsuperscript{10} Despite some initial controversy,\textsuperscript{11-13} this model stood the test of time, with the near-atomic resolution details being slowly revealed as cryo-electron microscopy techniques improved. The current model was determined at 3.6 Å by the Raunser group in 2016.\textsuperscript{14} At the time of the Holmes model, there was no reason to think that there would be more than a single filament architecture produced by actin-like sequences. The absolute conservation of eukaryotic actin filament structure likely arises from its role as an exceptionally highly connected hub,\textsuperscript{15} where the “universal-actin-pool” is harnessed by several filament nucleating machineries\textsuperscript{8,16} to provide force and architecture to a wide variety of biological processes. Essentially, once the eukaryotic actin filament was integrated into more than one biological process, a high degree of negative selection pressure restricted the actin sequence and structure to become frozen in time, since genetic drift\textsuperscript{17} favoring one biological process would have a negative impact on biological processes competing for actin.

**Prokaryotic actin-like filaments discovered**

The concept of there being a single actin began to change in the early 1990s, when the homologous structure of the non-polymerizing 70-kDa heat shock cognate protein was determined.\textsuperscript{18} Subsequent bioinformatics analysis predicted that eukaryotic actin is related to a larger family of proteins including hexokinase, heat shock proteins and the bacterial proteins FtsA, MreB and StbA/ParM.\textsuperscript{19} Similarity in these sequences was characterized by the conservation in amino acids participating in the ATP-binding pocket, later known as the actin fold.\textsuperscript{19,20} Yet it was not until 2001 that experimental evidence emerged that functional filament-forming actins exist in prokaryotes. These bacterial actins were subsequently termed actin-like proteins (ALPs).\textsuperscript{21}

Three common classes of ALPs have been identified in bacteria, with a forth being the rare MamK\textsuperscript{22} forming the scaffold of the magnetosome in magnetobacteria. MreBs form single stranded protofilaments, which can be arranged as antiparallel pairs, are present in most rod-shaped prokaryotes and are involved establishing cell shape.\textsuperscript{23} FtsAs contain a membrane inserting C-terminal amphipathic helix and recruit the cell division protein and tubulin homolog FtsZ to the mid cell surface via FtsZ’s C-terminal peptide. In concert both proteins self-organize into protofilament systems.\textsuperscript{24} MreBs and FtsAs are the most commonly found ALPs in prokaryotes. Lastly the plasmid segregating actins, the first characterized being ParM from the R1 plasmid in *E. coli* (*EcParM*).\textsuperscript{25} Together these ALPs all contain the conserved actin fold, but often with very low overall sequence identities of below 20%.\textsuperscript{26} Despite these large variations, all monomer structures solved to date by X-ray crystallography proved to be very similar.\textsuperscript{27}

**Variety in prokaryotic actin-like filaments**

Despite the structural similarity of the monomers, this did not prove true for the filament structures of ParM’s, which turned out to have huge variations. In the initial paper describing *EcParM*,\textsuperscript{25} this filament was thought to be just a small variation of F-actin in its helical parameters. Only after more extensive EM reconstructions did it become apparent that the structure differed substantially, in being a left-handed helical filament as opposed to the right-handed F-actin\textsuperscript{28} (Fig. 1). The evolutionary pressure that determined the handedness of the various biological filaments is not known at present. Other ParM’s investigated in the following years obtained by electron microscopy (AlfA from *Bacillus subtilis* plasmid pBET131 (*BsParM*), ParM from *Staphylococcus aureus* plasmid pSK41 (SaParM)) showed that the helical parameters could differ even more substantially from F-actin. Yet all these filaments (*EcParM*, *BsParM* and SaParM) were still polar double stranded straight helices, like F-actin.\textsuperscript{29,30}

The first departure from the dogma that all ParM’s formed only double stranded polar filaments came with the structure of Alp12 (*CtParM*) from *Clostridium tetani*, which segregates the pE88 plasmid encoding the lethal tetanus toxin.\textsuperscript{31} *CtParM* formed 4-stranded filaments (2 double strands) arranged in an open cylinder separated by a wide cleft (Fig. 1). Subunits within a single-stranded protofilament associated through subdomain interactions that have parallels to all other known actin structures.\textsuperscript{32} At the macroscopic level, subdomain 3 to subdomain 4 inter-subunit connections are the constant feature that lead to relatively unidirectional protofilaments,\textsuperscript{32} despite the lack of conservation of inter-subunit interacting residues or their positions within the protein sequences.\textsuperscript{33} Yet the 2 protofilaments formed a polar double-stranded filament through a completely different interface than observed for other actins. Although each double strand is polar, the 2 double stranded filaments are paired in anti-parallel fashion, by 2 β sheets of subdomain 3 to construct this novel open cylindrical architecture.\textsuperscript{31}

More recently, ParM from the pBMB67 plasmid in *Bacillus thuringiensis* (*BtParM*) was shown to form
supercoiled, rather than straight filaments, which although double stranded, are anti-parallel rather than polar (Fig. 1), a far departure from the construction of F-actin or any other ParM. ParM’s are plasmid segregation polymerizing actins that are linked to the parC regions of the plasmid DNA via adaptor proteins (ParRs), which are specific to each individual ParM/parC combination. Within the ParCMR system from E. coli (EcParCMR), the ParR/parC complex was shown to pair 2 or more filaments into randomly oriented bundles.

In this system other forces, such as cellular crowding, also paired EcParM filaments in the absence of the associated molecules. The roles of the 2 competing effects (direct binding of ParR/parC versus osmotic pressure) are difficult to separate, and hence their relative contribution is ambiguous. The ParCMR system from Bacillus thuringiensis (BtParCMR) proved to be entirely different and more amenable to teasing apart each contribution. Polymerization of BtParM in the presence of BtParR stimulated ATP hydrolysis by BtParM and formed a cylinder, comprised of 4 antiparallel strands, with inner and outer diameters of 57 Å and 145 Å, respectively, which is also formed in the presence of the BtParR/parC complex (Fig. 1). The structure of the BtParM cylinder is composed of 2 interwoven supercoiled antiparallel filaments, which is the geometry of the BtParM filament formed in the absence of ParR and parC (Fig. 1). Here osmotic pressure originating from molecular crowding also paired filaments, but in this case they form rafts of individual supercoiled filaments arranged in parallel (Fig. 2). Thus in the case of BtParCMR, the unique BtParM nanotubule geometry requires a second component of the BtParCMR system, ParR or ParR/parC.

Eukaryotic actin filaments function as in a universal-pool-of-actin in which variety in actin-binding proteins is able to harness the same molecular polymerizing motor for many cellular processes. Prokaryotic cells have developed different mechanisms for force generation. We have previously speculated that since bacteria have one-filament-one-function systems to create force through polymerization, they evolved distinct actin filaments to power specific functional processes within a single cell. This leads to several questions, firstly: Are there more novel actin filament architectures out there? Given the vast numbers of uncharacterized ParM’s that are known to exist from phylogenetic analyses, the answer can only be “yes.” A more pertinent question is: How much further will these filaments differ from F-actin? The answer to this question will likely be quickly
revealed by cryo-electron microscopy, which in just a few years has become the structural biology tool of choice to study filament systems at near-atomic resolution, due to the improvements in electron microscopes, and in particular, direct electron detectors. Finally, why did Nature create so many different designs of actin-like filaments for plasmid segregation?

The answer is probably for at least 2 reasons. Firstly, when 2 different plasmids reside in the same cell they will require different segregation systems to be faithfully inherited, hence, there may have been some positive evolutionary pressure for the ParMs to diversify. Secondly, if evolution is truly deterministic, the filament structures of plasmid segregating actin-like filaments are likely related to the sizes of the individual plasmids to be segregated. That is a ParM filament consisting of 4 strands (like CtParM) should be substantially stronger than a double stranded filament (like EcParM), thus being able to push a bigger load. This hypothesis is currently under investigation.

**Comparison of actin-like and tubulin-like filament structures**

Actin-like and tubulin-like filaments have transposable roles in biology. In prokaryotes, the tubulin-like protein FtsZ assembles to form the cytokinetic ring, whereas this function is performed by F-actin in eukaryotes. In contrast, plasmid DNA segregation is often orchestrated by ParM actin-like proteins in bacteria, while chromosome DNA segregation is choreographed by microtubules in eukaryotes. Comparison of the known structures formed by the actin and tubulin folds, indicates that these basic building blocks are capable of forming extensive ranges of structures. Both folds can form linear single stand filaments, twisted parallel pairs of filaments and tubules of various dimensions (Fig. 1). The ability to alter a tubule’s dimensions, though changing the number of strands in the tubule, has been demonstrated through protein engineering to require relatively minor surface amino acid modifications in the case of barrels formed from α-helices. Thus, various diameters of tubules formed from the actin and tubulin folds are likely to have been sampled during evolution. Whether a superior property is intrinsic to the actin fold for forming microfilament-like architectures, and to the tubulin fold for forming microtubule-like structures, or whether the use of these protein scaffolds were stochastic events that became entrenched in early eukaryotes remains open to debate. Nevertheless, it is clear that the highly specialized filament geometries of microfilaments and microtubules are just 2 of many possibilities that are available in nature.

**Conclusions**

The structures of F-actin and microtubules have been conserved over a billion years in eukaryotic cells. These structures are maintained through evolution by their interactions with large numbers of binding proteins, which have likely restricted their genetic drift and have allowed for the filament properties to be exploited by many cellular processes. In contrast, the one-filament-one-function design observed in many prokaryotic filaments has allowed for the adoption of a large variety of different filament structures. Despite the many variances in filament structures, 2 features are preserved between all acts. Firstly, the individual strands forming actin-like filaments (protofilaments) share grossly similar contacts, subdomains 1 and 3 from one monomer interact
with subdomains 2 and subdomain 4, respectively in the neighboring monomer in the protofilaments. Secondly the nucleotide-binding site, which accepts GTP in some ALPs, acts as a conformational switch activated by polymerization controlling ATP/GTP hydrolysis and phosphate release. The ATP/GTP switch (converting the initially bound ATP/GTP to ADP-Pi/GDP-Pi and subsequently to ADP/GDP) acts as a timing mechanism, which coordinates the depolymerization of actin filaments via the conserved contacts in the protofilaments.

The determinants as to whether filaments treadmill or are dynamically unstable largely remain unexplored for the ALPs. These activities will be impacted by: 1) off and on rates for each nucleotide-bound state of the monomers at both ends of the filament in its different nucleotide-bound forms; 2) nucleotide-exchange rates in monomers and at filament ends; 3) hydrolysis and phosphate-release rates on polymerization; 4) concentrations of ALPs and nucleotides; 5) higher order mechanisms for monomer association/dissociation with the filament; and 6) binding partners. A simple case for an in vitro dynamically unstable filament occurs when the nucleotide-exchange rate for monomers is slow relative to the off rate for ADP-bound protomers at the ends off the filament. On approaching steady state the filaments will depolymerize until the dissociated monomer pool has regenerated sufficient ATP-bound monomers to support repolymerization, as is suggested for EcParM. In contrast, in vitro treadmilling for actin results from the 2 ends of the filament having different on and off rates for monomers. However, these are just 2 of the multitude of possible scenarios.

Filament architectures reflect their function. MreB and FtsA filaments form non-helical protofilaments allowing them to present a consistent binding interface to the membrane, which would not be possible if they were twisted. ParM’s, in contrast, form 2 to 4 (and perhaps even more) stranded helical filaments. As motor proteins, the helical design brings greater rigidity to the filaments and by increasing the number of strands expands the possibilities to segregate larger payloads. The functional requirement of any ParM filament is to polymerize and interact with the specific plasmid through the complementary adaptor protein. This has allowed for far more latitude in exploring diverse filament architectures during evolution in comparison to filament systems that have more extensive interactions. Thus, we speculate that the currently known actin filament structures are likely to be a fraction of the total diversity.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.
