Liposome division by a simple bacterial division machinery

Masaki Osawa (大澤正輝)1 and Harold P. Erickson

Department of Cell Biology, Duke University Medical Center, Durham, NC 27710-3709

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We previously reconstituted Z rings in tubular multilamellar liposomes with FtsZ-YFP-mts, where mts is a membrane-targeting amphiphilic helix. These reconstituted Z rings generated a constriction force but did not divide the thick-walled liposomes. Here we developed a unique system to observe Z rings in unilamellar liposomes. FtsZ-YFP-mts incorporated inside large, unilamellar liposomes formed patches that produced concave distortions when viewed at the equator of the liposome. When viewed en face at the top of the liposome, many of the patches were seen to be small Z rings, which still maintained the concave depressions. We also succeeded in reconstituting the more natural, two-protein system, with FtsA and FtsZ-YFP (having the FtsA-binding peptide instead of the mts). Unilamellar liposomes incorporating FtsA and FtsZ-YFP showed a variety of distributions, including foci and linear arrays. A small fraction of liposomes had obvious Z rings. These Z rings could constrict the liposomes and in some cases appeared to complete the division, leaving a clear septum between the two daughter liposomes. Because complete liposome divisions were not seen with FtsZ-mts, FtsA may be critical for the final membrane scission event. We demonstrate that reconstituted cell division machinery apparently divides the liposome in vitro.

Bacterial cytokinesis is generated by a complex ring-shaped structure comprising more than a dozen proteins. It is called a Z ring because the primary cytoskeletal framework is made by filamentous temperature-sensitive Z (FtsZ). FtsZ has a globular core homologous to tubulin, and at its C terminus there is an ∼50 amino acid unstructured peptide linker, followed by an ∼17 amino acid peptide that binds FtsA (see ref. 1 for a review of FtsZ). FtsA has a C-terminal amphipathic helix that inserts into the membrane, and it therefore serves to tether FtsZ to the membrane. FtsA is also the primary dock for most of the downstream division proteins. FtsA proteins from Streptococcus pneumoniae and Thermotoga maritima have been shown to assemble into actin-like filaments in vitro (2, 3). FtsA from Escherichia coli has been described as “recalcitrant” and difficult to work with in vitro (4). However, a mutant termed FtsA* has been shown to facilitate cell division in vivo (5, 6) and to be amenable to in vitro experiments (7).

We previously achieved the reconstitution of Z rings in vitro using an engineered construct FtsZ-YFP (yellow fluorescent protein)-mts, where mts is an amphipathic helix that can tether FtsZ directly to the membrane, bypassing FtsA (8). The Z rings assembled spontaneously when FtsZ-YFP-mts was incorporated with GTP into multilamellar tubular liposomes, and they generated a constriction force without the need for any other protein. The constriction force generated invaginations of the liposome wall but not a complete septation. The lack of complete septation may be due to the thickness of the wall in the multilamellar liposomes and/or an intrinsic inability of FtsZ to the membrane? (iii) Can we achieve complete division and septation of liposomes?

Results

We used the previously developed emulsion method (9) to obtain unilamellar spherical liposomes with FtsZ (and FtsA* when wanted) inside (Fig. S1 A and B). However, there were problems in observing the structures in the light microscope when suspended in a simple aqueous solution. First, liposomes floating in solution were constantly moving, making it difficult to photograph. Second, liposomes often burst when they contacted the glass surface. Third, it was difficult to achieve distortion into a tubular shape, which is preferable for formation of Z rings. Although several sophisticated methods have been proposed using laser tweezers and/or micro-manipulators (10, 11), we found that simply mixing the liposomes with a low concentration of agarose resolved all of these problems. We mixed the liposome solution with agarose immediately after formation by the emulsion method. Because the low concentration of agarose hardened slowly at room temperature, the mixture easily spread in the narrow space between the slide glass and the coverslip. When the agarose hardened, the liposomes were motionless, and clear images could be acquired (Fig. 1). The shear induced during the spreading distorted the liposomes into a variety of shapes, including spherical and tubular shapes as shown below.

Z-Ring Assembly and Membrane Bending by FtsZ-YFP-mts. We first applied this agarose method to FtsZ-YFP-mts inside large spherical unilamellar liposomes. We were able to acquire much clearer images of the patches of FtsZ-mts on the inner surface of liposomes (Fig. 1 A) compared with those without agarose (Fig. S1 D). These patches depended on FtsZ polymerization, as we previously described (Fig. S1 C). When imaged at the equator of the liposome, the patches coincided with bending distortions of the membrane (Fig. 1 A, arrows). The bending produced projections toward the outside of the liposome, which corresponded to concave depressions, as viewed from the inside of the liposome, similar to the concave depressions formed on the outside of liposomes in our previous study (12). This correlation is more easily seen in liposomes with a small number of patches (Fig. 1 A’ and A”). Importantly, when we imaged these patches en face, on the upper surface of large spherical liposomes, we saw that many of them had the shape of a small rings, ranging from 600 to 1,500 nm in outside diameter and with a hole in the center. The frequency of these rings in the en face section suggests that many of the patches seen at the equator (Fig. 1 A) are rings seen edge on. We also found some smaller dots, which may be small rings where the central hole is not resolved. In addition to the rings, there were some...
amorphous patches similar to those we have seen before on the outside surface of liposomes (Fig. 1 B and C, arrowheads) (12).

Some patches were elongated and may be precursors of longer bundles that develop under some conditions (Fig. 2). These small Z rings appear to maintain the membrane bending into concave depressions. One possible scenario is that the FtsZ initially forms patches, with short protofilaments running in several directions, and these initiate the bending into concave depressions. These patches then reorganize into rings that run around the periphery of the depression and maintain its bending.

Fig. 1D shows a small vesicle with a single Z ring, appearing as two bright dots (the Z ring seen in projection at the edge) connected by a thin line. This Z ring appears to constrict from 0 to 80 s, but it does not constrict the vesicle. Rather it seems to slide along the side of the vesicle to achieve the smaller diameter. Its more constricted form may approach the structure of the small rings seen en face in Fig. 1 B and C, although it is not producing a concave depression.

Some of the liposomes adopted thin, tubular extensions, in which we often found Z rings, generally at sites of constriction (Fig. 1E). In the most constricted rings, we could not resolve the gap of the membrane wall, indicating that the diameter of the constriction is less than the 250-nm resolution of the light microscope (Fig. 1G). The fluorescence from FtsZ-mts was exactly localized as a spot at the constriction site. We also found skinny unilamellar tubular liposomes that had multiple small Z rings. These Z rings were localized at kissing points of the membrane constrictions in the differential interference contrast (DIC) image (Fig. 1G; Fig. S2). Therefore, we think that Z rings produced by FtsZ-mts are able to constrict to less than 250 nm, but may not achieve full division.

Z-Ring Assembly, Vesicle Constriction, and Complete Septation by FtsZ Plus FtsA*. To reconstruct more natural Z rings in liposomes, we turned to a mixture of FtsZ and FtsA, which is the native membrane tether for FtsZ (13). We were unable to obtain useful reconstitutions using WT FtsA. The mutant FtsA* (5, 7, 14) gave much better results as an expression protein and was used for all studies. FtsZ-YFP (which has YFP following the C-terminal, FtsA-binding peptide) showed a variety of localization patterns when assembled inside liposomes with FtsA*, ATP, and GTP. In 2.5% of liposomes, FtsZ associated with the membrane, most likely
through FtsA*, and was evenly distributed on the membrane, with some more intense foci (Fig. 2A). In the same conditions, 14% of liposomes showed FtsZ assembled into bundles (Fig. 2B and C). These bundles often sprouted from foci, which might be nucleation centers of FtsZ filaments. The bundles grew into the liposome interior (Fig. 2B) or along the liposome membrane (Fig. 2C). In some liposomes, these bundles connected with each other and formed net structures on the inner surface of liposomes (Fig. 2D and E). These net structures formed by FtsZ bundles did not show constrictions and may have a substructure different from Z rings. Without GTP, we did not see any significant structure except some dots or rarely small rod-like structures on the membrane.

A small fraction of liposomes had Z rings that were localized at constriction sites. Some of these Z rings actually showed continued constriction in time-lapse recordings. Our best example is shown in Fig. 3. A large diameter Z ring, which was initially located at the moderately constricted site, continued to constrict and developed a highly constricted invagination within several minutes. At 488 and 603 s, the DIC images still show a narrow gap between the invaginating edges, and at 510, 522, 630, and 682 s, the fluorescence shows the two-dot structure characteristic of a Z ring. At 742 s, however, the two-dot structure has dispersed and shifted to one side, and at 777 s, the DIC image shows that the gap has been replaced by a continuous septum. This liposome appears to have completed division. Probably the division was completed between 682 and 742 s. Two additional examples of apparent complete division are shown in Figs. S3 and S4 and are described in the figure captions.

Quantification of results has been difficult because of the low frequency of Z rings assembled in liposomes and their highly dynamic property. However, in one large experiment, we found 42 Z rings and followed them over time, with the following results. Fourteen Z rings constricted to apparently complete septation; 14 Z rings constricted the liposomes but not to complete septation; and 24 Z rings were located at a visible constriction site that did not change their diameters during the ~20 min of observation. In another large experiment, we checked 400 liposomes to see if Z rings were formed. These liposomes were checked within 20 min after starting the reaction in order not to miss Z rings that had already progressed to a high level of constriction. We found eight Z rings, meaning that 2% liposomes had a Z ring. Therefore, 1.3% liposomes (14 + 14)/42 × 2% showed progressive constriction during the observation.

To thoroughly confirm that the Z ring is generating the liposome division, we followed negative controls containing FtsZ and FtsA* but no GTP. We imaged more than 200 liposomes for 1 h. Most liposomes showed a static structure, and a few showed small shape changes, examples of which are shown in Fig. S5. The clearly progressing constrictions or divisions as shown in Fig. 5 and Figs. S3 and S4 were never observed, indicating that liposome constrictions and division are generated only by active Z rings, requiring GTP.

When GTP was replaced with the GMPCPP, a slowly hydrolyzable GTP analogue, we found large foci but no structures like Z rings. We then tested the effect of omitting ATP, which is required for FtsA* assembly (2, 3). Recruitment of FtsZ-YFP to the membrane was reduced without ATP, perhaps because the affinity of monomeric FtsA* to the FtsZ filaments and/or the membrane is lower than that of polymerized FtsA. We did not find any Z rings in liposomes in the absence of ATP.

**Discussion**

Using our unique system to observe Z rings in unilamellar vesicles, we successfully addressed the three questions raised in the Introduction. (i) The membrane-targeting FtsZ construct FtsZ-YFP-mts assembled patches on the inside of the liposome. When the patches were viewed en face, on the top surface of the larger vesicles, many appeared to be small Z rings, ~600–1500 nm in diameter, parallel to the plane of the membrane. These small Z rings did not achieve division but seemed to generate concave bending of the membrane, as viewed from the inside of the liposome. Additionally, the FtsZ-YFP-mts appeared able to generate Z rings that constricted when confined to smaller diameter tubular liposomes. (ii) The natural, two-protein system of FtsZ plus FtsA* produced small patches and elongated bundles of FtsZ attached to the membrane, presumably tethered via the FtsA*. (iii) Most importantly, the FtsZ-FtsA* was able to produce Z rings that encircled liposomes and occasionally achieved complete constriction and septation.

Reconstitution of Z rings in giant unilamellar vesicles, using the two-protein system FtsA and FtsZ, has been attempted previously (15). FtsA alone localized to the walls of the liposome; FtsZ alone formed small clumps and some fibers, dispersed in the vesicle interior and adherent to the membrane. When FtsZ and FtsA were mixed, they colocalized to fibers and clumps in the interior of the vesicle. The FtsZ appeared to dislodge the FtsA from the membrane. The failure to form Z rings in this study might be attributed to a number of experimental differences. The previous study used wild-type FtsA rather than the more tractable mutant FtsA* that we found important. It used a fivefold excess of FtsZ, whereas we had the best results with from half to equimolar FtsZ against FtsA. Finally, it incorporated 50 mg/mL Ficoll in the reconstitutions, a crowding agent that may cause the FtsZ protofilaments to associate into bundles. These bundles, sometimes appearing as clumps, may have prevented the assembly of the much thinner ribbons of protofilaments that constitute Z rings.

Although both FtsZ-YFP-mts and FtsZ-YFP/FtsA* formed Z rings in unilamellar liposomes and generated constriction force, they behaved differently. First, unlike FtsZ-YFP-mts, FtsZ-YFP/FtsA* did not form multiple concave depressions and small Z rings on the surface of large liposomes. Second, complete division was not observed with the membrane-targeted FtsZ. These results suggest that the FtsA* plays a role in organizing Z-ring assembly and in the final scission event. These events may involve assembly of FtsA* into polymers on the membrane, which serve to organize FtsZ protofilaments (2, 3). Another possibility is that the bond between FtsA* and the C-terminal peptide of FtsZ, which was reported to be very weak, Kd ~ 50 μM (3), may be more readily reversible than the direct insertion of the mts of FtsZ-YFP-mts into the bilayer. This weak interaction may permit the disassembly of the Z ring in the final stage of division. This situation may also explain why FtsZ-YFP-mts
can form multiple concave depressions and Z rings on the almost flat surface of large liposomes, whereas FtsZ-YFP/FtsA* cannot.

There are several possible reasons for the low frequency of Z-ring assembly and constriction. In our liposome system, it takes about 10 min from the time of liposome formation to observation under the microscope. Some liposomes might have finished division within this period. However, the most likely impediment is the size and shape of the liposomes, which was not easily controlled in our soft agarose gels. Many of the liposomes were up to 50 μm in diameter, which is much larger than the maximum diameter of ~5 μm that we had previously observed for Z-ring assembly in tubular liposomes (8). The shape of the liposome is probably also an important factor, with an elongated shape being more favorable for Z-ring assembly, constriction and division.

The surface area of each liposome in our system is fixed. If a spherical liposome divided into two equal spheres, the total surface area would have to be equal to that of the mother liposome. This situation would require that the total volume of the daughter liposomes be reduced by 30%. Because the lipid bilayer is impermeable to salt, this would cause a large increase in osmotic pressure in our buffer condition. This increase may stall Z-ring constriction. However, liposomes with an elongated or tubular shape may avoid the osmotic pressure increase: one precise tubular geometry can divide into two identical spheres conserving both total surface area and total volume (16). In this case, the Z ring would only need to generate a force sufficient to bend the membrane. In our system, the agarose gel may impose additional constraints. If division took place after the agarose gel had hardened, the expanding daughter spheres/shrinking parental tube would need to compress/stretch the gel, and this would require significant force. It is likely in our experiments that the initial constriction and accompanying shape change takes place before the gel hardens, minimizing the need for this extra force. We note that the liposomes are frequently not perfect spheres but asymmetric (Fig. S4), which could allow progression of the septum with minimal further compression/stretching of the gel.

The constraints of constant surface area and volume are averted in living cells because they are able to assemble new membrane that can accommodate the increased volume, as well as the formation of a septum. Indeed, there are certain cells that normally use FtsZ, but can be converted to cell wall-less L forms, which achieve division in the complete absence of FtsZ (17, 18). We have suggested that these divisions may be affected by an excess production of cell membrane, which could produce invaginations that eventually fuse to divide the cell (19). Recent studies have shown that membrane fluidity plays a crucial role in the division of these L forms and suggested that biological processes of the membrane alone might have been sufficient for the division of primitive cells (20, 21). The FtsZ-FtsA system greatly increases the efficiency of the process and is able to achieve division in the absence of excess membrane synthesis.

Materials and Methods

Protein Preparation. The C-terminal YFP (Venus) fusion of FtsZ (FtsZ-YFP) and membrane targeted FtsZ (FtsZ-YFP-mts) were prepared as previously described (8, 22). Briefly, the soluble fraction of FtsZ-YFP or FtsZ-YFP-mts was precipitated with 30% saturated ammonium sulfate followed by anion exchange chromatography on a resource Q column.

FtsA-R286W (FtsA*) was expressed in C41 cells (derivative of BL21 (23)) from pWM1690, which was provided by William Margolin (University of Texas-Houston Medical School, Houston, TX) (7). This plasmid adds a His6-T7 tag at the N terminus. An optical density at 600 nm of 0.5, cells were induced overnight with 1 mM isopropyl beta-D-1-thiogalactopyranoside (IPTG) at 37 °C. Cells were collected by centrifugation and suspended in buffer A (50 mM Tris-HCl, pH 7.9, 50 mM KC1, 10% glycerol, 1 mM EDTA) with 400 μg/mL lysozyme. After two cycles of freeze-thaw, the cells were sonicated followed by centrifugation for 20 min at 32,000 rpm (rotor Ti41.2; Beckman). The pellet was suspended in buffer B (90 mM Tris-HCl, pH 7.9, 350 mM KC1, 10% glycerol), containing 1% Triton X-100. This suspension was centrifuged for 20 min at 32,000 rpm (rotor Ti41.2; Beckman), and the supernatant was collected. The supernatant was applied to a Talon column, washed with buffer B containing 1% Triton X-100, and then extensively washed with buffer B without Triton X-100. FtsA* was eluted with buffer B containing 160 mM imidazole. The total yield was about 10 mg FtsA* from 1 L bacterial culture. DTT was added to 0.1%, and the protein was stored at −80 °C.

All proteins were dialyzed in polymerization buffer, HMCKG [50 mM HEPES/KOH, pH 7.7, 5 mM MgAc2, 300 mM KC1, 50 mM KC1, 10% (vol/vol) glycerol] containing 0.1% DTT. FtsA* was centrifuged after dialysis to remove precipitated protein. FtsA* lost activity after multiple freeze-thaw cycles in HMCKG. Protein concentrations were determined by bichinonic acid (BCA) assay using BSA as a standard for FtsA*. YFP absorption was used to determine concentrations of FtsZ-YFP and FtsZ-YFP-mts as previously described (22).

Liposome Preparation and Observation. We used the emulsion method to prepare unilamellar liposomes (9). Phosphatidylcholine (PC) and 1,2-dioleoyl-sn-glycerol-3-phospho-rac-(1-glycerol) (DOPG) dissolved in methanol at 10 mg/mL were mixed at a 4:1 volume ratio. Twenty-five microliters of the mixture in a microtube was dried with air current followed by the addition of 250 μL of mineral oil. To disperse phospholipid in mineral oil, the mixture was sonicated 20 times for 2 s and then left at room temperature for 5 h. The FtsZ-YFP/FtsA* assembly reaction was prepared in a microtube. The typical condition was 7 μM FtsZ-YFP, 7 μM FtsA*, 2 mM GTP, 0.5 mM ATP, and 0.05% DTT in HMCKG. We placed 5 μL of this reaction solution in 125 μL of the mineral oil and phospholipid mixture described above and mixed vigorously to form an emulsion. This emulsion mixture contained small aqueous micelles of FtsZ-YFP and FtsA, surrounded by a lipid monolayer and suspended in mineral oil. This 130-μL oil suspension was layered on 50 μL of HMCKG buffer as an external feeding solution, followed by low speed centrifugation (1400 g for 3 min; Costar microcentrifuge). When they passed through the lipid monolayer at the boundary between the oil and aqueous phases, the micelles became unilamellar liposomes, with a single lipid bilayer containing FtsZ-YFP and FtsA* (9). Twenty microliters of 1% agarose in HMCKG at 60–65 °C was added to 50 μL of the unilamellar liposome preparation and quickly mixed before placing on a glass slide. A 22 × 60-mm coverslip was put on top to spread the solution it before it hardened. If the 7 μM FtsZ-YFP is hydrolyzing GTP at 5 s per min, the 2 mM GTP would last about 60 min. The liposomes were observed by DIC and fluorescent microscopy as previously described (12). Focus was adjusted in DIC before taking fluorescent images.

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Supporting Information
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Fig. S1. Unilamellar liposomes produced by the emulsion method, observed without agarose. (A) DiI, a fluorescent lipid dye, was dissolved in methanol with phospholipids [DiI:egg phosphatidylcholine (PC):1,2-diOleoyl-sn-glycero-3-phospho-(1-glycerol)] (DOPG) = 0.25:200:50 μg], and unilamellar liposomes were formed by the emulsion method as described in Materials and Methods. (B) Filamentous temperature-sensitive Z (FtsZ)-yellow fluorescent protein (YFP) (7 μM) and 1 mM GTP are inside liposomes. FtsZ-YFP (with no mts) is diffusely distributed. (C) FtsZ-YFP-mts (7 μM), where mts is a membrane-targeting amphiphilic helix, in the absence of GTP. There is a slightly enhanced YFP signal at the liposome membrane (arrow). (D) FtsZ-YFP-mts (7 μM) in the presence of 1 mM GTP. Patchy structures with concave depressions (from the inside of liposome are seen; arrows). In all images, the left panels are fluorescence images (a rhodamine filter cassette was used for A and a YFP filter cassette for B–D) and the right panels are DIC images. (Scale bar, 10 μm.)
Fig. S2. A skinny tubular liposome has several Z rings at constriction sites whose diameters are close to or less than the resolution of the light microscope. (Upper) YFP fluorescence. (Lower) DIC. White bars show localizations of Z rings at membrane kitting points in the DIC image. (Scale bar, 10 μm.)

Fig. S3. Time lapse recording of small liposome division with FtsZ-YFP plus FtsA*. The recording started with a Z ring (arrow) at a preformed constriction at time 0 (see DIC image at 61 s). The constriction deepens over time. A gap can still be resolved at 144 s (arrow), and fluorescence shows a two-dot structure at 163–222 s. At 246 s, the DIC shows the gap replaced by a straight line (septum), and fluorescence shows a single diffuse stripe from 268 s onward. At 416 s, this fluorescence has dissipated, and at 449 s, DIC shows a clear complete septum. (Scale bar, 10 μm.)

Fig. S4. Time lapse recording of a uniquely asymmetric liposome division with FtsZ-YFP plus FtsA*. The Z ring initially has a bright dot on the left side (which is also clearly visible in DIC (arrows at 0 and 20 s), and the constriction is deep on the left side, whereas the right side remains straight from 366 to 754 s. At 1,495–1,825 s, this flat membrane seems pulled toward the invaginating septum. In this time frame the DIC shows the septum to be a deep V, which is closing at later times. The septum has a bright dot at its tip, which is still pretty clearly separated from the flat membrane. Suddenly, at 1,882 s, the V-shaped septum collapses into a single line, and the bright dot seems transferred to the flat membrane on the right. This transfer is reflected in the fluorescence, where the two-dot structure is bright on the septum side at 1,527 and 1,792 s but bright on the flat-membrane side at 1,906 s. The appearance of a complete septum is maintained at 1,946 s.
A negative control experiment showing liposome shapes without FtsZ assembly. As described in Materials and Methods, the reaction mixture for liposome division contains FtsZ-YFP, FtsA*, ATP, and GTP. To eliminate FtsZ assembly for this negative control, GTP was removed from the reaction mixture. More than 200 liposomes were observed at time 0 and 1 h later. Selected images with the most interesting shapes are shown here. The first panel shows a DIC image of the liposome at time 0. The second panel shows the fluorescence from FtsZ-YFP at time 0. The third and fourth panels show the same liposome 1 h later. (A–G) Various shapes of liposomes are shown, none of which significantly changes shape. Different sizes of spherical liposomes (A–C) and cylindrical liposomes (D and E) are shown. F and G show liposomes of irregular shape, which are probably formed by shear force when the reaction mixture spreads on the slide glass. Interestingly, these shapes are maintained even after 1 h, suggesting they are molded by the soft agarose. (H–J) Occasionally we found liposomes that changed their shape. The liposomes in H and I elongated slightly, whereas that in J shortened. In the 200 control images without GTP, we never found liposomes with a significant constriction, and nothing indicating a constriction progressing during the 1-h observation. The FtsZ-YFP was mostly diffuse in the vesicle, but bright foci were observed in 50% of liposomes, suggesting that clustered or condensed FtsA may recruit monomeric FtsZ-YFP.