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ABSTRACT

Intercellular fluids in living organisms contain high concentrations of macromolecules such as nucleic acid and protein. Over the past few decades, several studies have examined membraneless organelles in terms of liquid–liquid phase separation. These studies have investigated aggregation/attraction among a rich variety of biomolecules. Here, we studied the association between the polymerization/depolymerization of actin, interconversion between monomeric (G-actin) and filamentous states (F-actin), and water/water phase separation in a binary polymer solution using polyethylene glycol (PEG) and dextran (DEX). We found that actin, which is a representative cytoskeleton, changes its distribution in a PEG/DEX binary solution depending on its polymerization state: monomeric G-actin is distributed homogeneously throughout the solution, whereas polymerized F-actin is localized only within the DEX-rich phase. We extended our study by using fragmin, which is a representative actin-severing and -depolymerizing factor. It took hours to restore a homogeneous actin distribution from localization within the DEX-rich phase, even with the addition of fragmin in an amount that causes complete depolymerization. In contrast, when actin that had been depolymerized by fragmin in advance was added to a solution with microphase-separation, F-actin was found in DEX-rich phase droplets. The micro-droplets tended to deform into a non-spherical morphology under conditions where they contained F-actin. These findings suggest that microphase-separation is associated with the dynamics of polymerization and localization of the actin cytoskeleton. We discuss our observations by taking into consideration the polymer depletion effect.

I. INTRODUCTION

Inside living cells, various solutes and biological factors, including macromolecules such as nucleic acid and protein complexes, are present in high concentrations. It has been reported that liquid–liquid phase separation (LLPS) triggered by this very congested environment plays an important role in driving a rich variety of cellular activities. To date, the mechanisms that maintain a variety of multi-step, but robust, regulatory systems that are often found in cells, trigger the onset of diseases caused by

proteins essential for cells, or sense physical signals, such as temperature, have been difficult to explain solely in terms of individual biological molecules. However, these phenomena are now understood in terms of the spatial sorting of biological factors by LLPS or the physicochemical dependency of LLPS.[1–5] In particular, it is now considered that droplets that behave like a solution, within which proteins, RNA, and other biomolecules are condensed, are formed through attractive interactions among these factors affected by LLPS. Non-membranous intracellular structures, i.e., nucleoli, stress granules, and P-bodies, without lipid bilayer membranes are liquid-phase condensates and are considered to be formed through the LLPS process mentioned above.[6,7] This notion could be examined through the use of an aqueous two-phase system (ATPS) containing soluble polymers with various structures and properties, such as polyethylene glycol (PEG) and dextran (DEX). Using an ATPS, researchers have attempted to construct a model of the cellular structure in a self-organized manner,[8–11] which includes membraneless microcompartments in cells.[12]

Actin is a 42 kDa protein that is called G-actin when it is in a monomeric state and F-actin when it is polymerized and in a filamentous state. F-actin itself behaves as a smart natural polymer that has a variable length; it can form bundles or networks by crosslinking factors and is involved in the generation of force and movement by its own treadmill or in collaboration with the molecular motor myosin. As a cytoskeleton protein, it plays central roles in a wide range of cellular functions, including cell morphogenesis, cell motility, cytokinesis, and transport or anchoring of various intercellular cargo.[13] In most cases, actin dynamics through depolymerization and polymerization exhibit an indispensable role.

Various studies have revealed the behavior of actin in cell-like models such as liposomes or water-in-oil droplets.[14–20] Here, to investigate the effect of phase separation that causes micro-segregation, the behavior of actin in a binary solution of PEG and DEX was observed.[21,22] The results showed that G-actin spreads homogeneously throughout the solution, whereas F-actin was spontaneously distributed in DEX-rich phase droplets. Such droplets containing F-actin tended to be non-spherical. Moreover, it was shown that actin polymerization might be promoted in a solution that causes LLPS. It is known that actin polymerizes in the presence of a certain concentration of salt, and F-actins are bundled at a Mg^{2+} concentration higher than 10 mM. However, in a binary solution, actin could be polymerized at a lower salt concentration, and F-actins are bundled at a lower Mg^{2+} concentration.

In the present study, by observing the behavior of actin that was added to a PEG/DEX binary solution together with a protein that helps regulate the polymerization/depolymerization of actin, we investigated whether the regulatory system functions as in the bulk solution even under conditions that lead to phase separation, i.e., even in crowded environments like the cell interior. Fragmin used in this study is a protein with a molecular weight of 42 kDa that belongs to the gelsolin superfamily. The gelsolin superfamily protein is found in all eukaryotes as a representative factor that regulates the polymerization state of actin; it causes the depolymerization of F-actin in the presence of Ca^{2+} by severing a filament and continually capping its fast-growing end (barbed-end).[23,24]

II. MATERIALS AND METHODS

A. Polymers and reagents

We used a polyethylene glycol (PEG)/dextran (DEX) aqueous two-phase system (ATPS). PEG 6000 was purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan); its average molecular weight (MW) was 7300–9300 Da. DEX was also purchased from FUJIFILM Wako Pure Chemical Industries; its average MW was 180 000–210 000 Da. These two polymers were dissolved in Milli-Q water (18.2 MΩ cm) to prepare 20 or 30 wt. % stock solutions. For use as a tracer for PEG-rich domains, methoxyl PEG fluorescein (mPEG-Fluorescein) was purchased from Nanocs, Inc. (New York, NY); it had an average MW of 10 kDa, an excitation wavelength (Ex) of 490 nm, and an emission wavelength (Em) of 520 nm. It was dissolved in Milli-Q water to give a 10 wt. % stock solution. In the preparation of each sample, a mixture of PEG and DEX was agitated just before mixing with the other solutions.[25]

Other reagents of special grade or higher for biochemistry were purchased from FUJIFILM Wako Pure Chemical Industries and dissolved in Milli-Q water as stock solutions.

B. Proteins

Actin was prepared from chicken breast muscles or rabbit skeletal muscles according to the method by Spudich and Watt, except that the tropomyosin–troponin complex was removed before preparing the acetone powder.[26] Actin was labeled with fluorescent dyes (Alexa 488 maleimide or Alexa 546 succinimidyl ester), as described previously.[27,28] Otherwise, to visualize actin, rhodamine–phalloidin (R415, Thermo Fisher Scientific, Waltham, MA, USA) was added to F-actin before it was, in turn, added to PEG and/or DEX solutions.[22] Unless noted otherwise, the latter was used for fluorescent labeling of actin.

In general, G-actin was stored in a salt-free buffer [0.1 mM ATP, 1 mM $NaHCO_3$, and 0.1 mM $CaCl_2$ with or without 2–5 mM Tris-HCl (pH 8.0)], and F-actin was polymerized in a salt-containing buffer [5 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.2 mM ATP, and 1.0 mM $MgCl_2$]. The concentration of sodium bicarbonate or calcium chloride was sometimes slightly different, but this did not affect the results if the difference was within 10%. The concentration of the reducing agent was changed as appropriate based on the results obtained. The samples of F-actin were kept on ice until use.

Fragmin derived from the slime mold *Physarum polycephalum*, which was synthesized and purified using an expression system in *Escherichia coli*, was dissolved in the fragmin buffer [2.0 mM Tris-HCl (pH 8.0), 1.0 mM EGTA] and stored on ice until use.[29,30] Notably, the severing and barbed-end-capping activities of fragmin against F-actin are not affected by phalloidin.[31]

The distribution of fragmin in the PEG/DEX binary solution was examined as follows: After the binary solution containing fragmin completely separated into two layers, i.e., the upper PEG-rich and the bottom DEX-rich phases, equal-volume aliquots from the two phases were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The amount contained in each phase was then obtained from the density of the protein band.

C. Microscopy

Images were obtained with phase contrast and fluorescence microscopes (BX60/IX70, Olympus, Tokyo, Japan) with a 40× objective (NA = 0.75, Olympus) attached to a polarizing unit and a camera (WAT-910HX, Watec, Tsuruoka, Japan/IR-1000, DAGE-MTI, Michigan City, IN, USA). The acquired images were recorded in a hard disk by a computer through an image capture unit (DFG/USB2aud, Imaging Source, Bremen, Germany). The program ImageJ (http://imagej.nih.gov/ij/) was used to adjust the contrast and analyze images.[22]

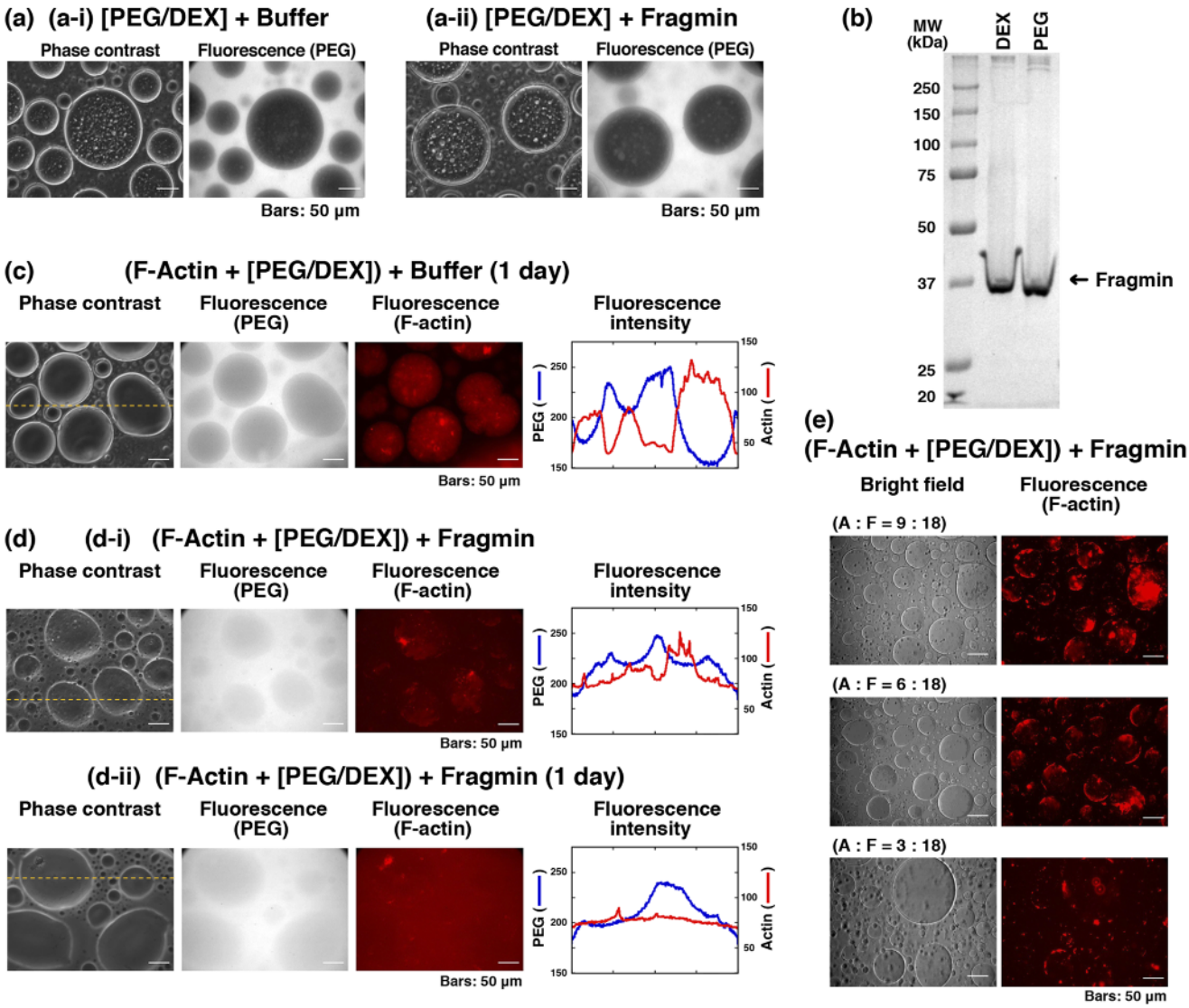

FIG. 1. The effect of fragmin on F-actins in the PEG/DEX binary solution. (a) Control experiments for the samples several minutes after the preparation of the PEG/DEX binary solution in the absence of actin, together with buffer (a-i) in the absence and in the presence of fragmin (a-ii), respectively. For each condition, phase contrast (left) and fluorescence images of PEG (right) are represented. (b) The distribution of fragmin in a PEG/DEX binary solution as examined by SDS-PAGE. The solution conditions were the same as in (a-ii). The concentration ratio (PEG/DEX) obtained from the density of the protein band of SDS-PAGE was 0.99 ± 0.06 (Average ± S.D.) (c) and (d) Actin distribution in the PEG/DEX binary solution after addition of the buffer (c) or fragmin (d). From left to right, the phase contrast image, fluorescence image of PEG, and fluorescence image of F-actin are shown. The right graph shows the relative fluorescence intensities (arbitrary unit) of PEG (blue line) and actin (red line) obtained corresponding to the yellow broken line in the field of view in the phase contrast images. Images in panels labeled "1 day" were obtained after 24 h [(c) and (d-ii)]; otherwise, images were obtained several minutes after the samples were prepared. Even when the same sample was observed, the position of the observation field was not always the same. The fluorescent images of F-actin shown here and in the following figures are pseudo-colored in red. (e) Dependence on the concentration ratio of actin and fragmin. Bright field (left) and fluorescence images of F-actin (right). This observation was performed under condition II (see Sec. II C). The final concentration of fragmin was 18.0 μM in all cases. The final concentration of F-actin was 9.0 (upper), 6.0 (middle), and 3.0 μM (bottom). Thus, the molar ratios of actin and fragmin (indicated as "A:F") were 9:18, 6:18, and 3:18, respectively.

The solutions prepared as described above were mixed in the order, PEG/DEX, actin, and fragmin or actin, fragmin, and PEG/DEX, in each microchamber made from a slide glass and a double-sided seal (SLF0601 Frame-Seal 15 × 15 mm^2 65 μl, BIO-RAD, Hercules, CA, USA) and then sealed with a coverslip, prior to microscopic observations. The final conditions of the sample solution other than proteins were as follows: condition I: 5.3 mM Tris-HCl (pH 8.0), 0.1 M KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 0.14 mM EGTA, 0.2 mM ATP, 5% PEG (or 4.9% PEG and 0.1% mPEG-Fluorescein), and 5% DEX or condition II: 7 mM Tris-HCl (pH 8.0), 0.1 M KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 1.0 mM EGTA, 0.2 mM ATP, 5% PEG, and 5% DEX. The final concentrations of added F-actin and fragmin were both 7.0 μM. Experiments with condition II or with protein at a concentration different than that mentioned above are indicated in the figure caption. Unless stated otherwise, all of the reported results are based on at least three independent experimental runs.

III. RESULTS

A. F-actin distribution in the presence of fragmin

As a control experiment, to confirm that the addition of fragmin has no effect on LLPS, fragmin or only the buffer used to make the fragmin stock solution was added to the PEG/DEX binary solution in the absence of actin. Phase separation, which is associated with the morphology and quantity of DEX-rich phase droplets in the PEG/DEX binary solution, in the presence of fragmin was indistinguishable from those before the addition of fragmin or after the addition of the buffer alone [Fig. 1(a)]. Compared with the experiment using Milli-Q water, when a buffer containing salt was used, there was a tendency for more PEG-rich droplets to temporarily enter DEX-rich droplets. However, essentially no change was observed in the phase separation itself even after the solution was left to stand for a day. In addition, fragmin was distributed in both PEG and DEX phases at equal concentrations [Fig. 1(b)].

As in a previous study, in a binary solution that causes LLPS, F-actins were distributed only within the DEX-rich phase [Fig. 1(c)].[22] F-actins often formed tangled aggregates or bundles inside the DEX-rich phase droplets or formed bundles beneath the interface of DEX-rich phase droplets. After the solutions were left to stand for a day, no significant change occurred in the DEX-rich phase droplets. The tendency of F-actin to form an aggregate or bundle appears to be greater than that in a previous study, perhaps because the solution had been adjusted, such as by the addition of calcium ions, so that fragmin is active.

Upon the addition of fragmin, actin, which was only localized in DEX-rich phase droplets, was also present in the PEG-rich phase [Fig. 1(d)]. The results did not depend on whether fluorescent labeling consisted of the addition of rhodamine–phalloidin or covalent modification with Alexa (Fig. S1). To make actin distribute evenly throughout the binary solution that causes LLPS, it took a long time, from at least three hours to about one day (Fig. S2), even with fragmin in an amount that can completely depolymerize F-actins within several minutes in the bulk solution.

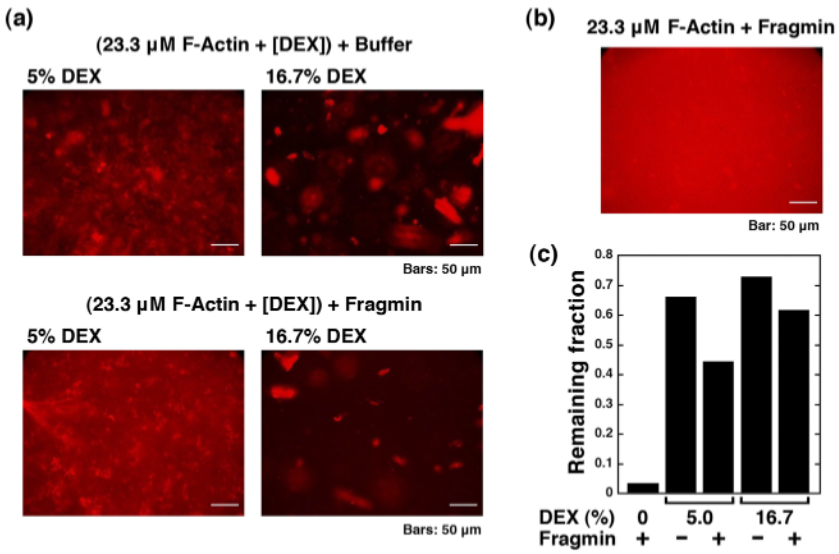

FIG. 2. Protection of F-actin against the depolymerizing activity of fragmin in the presence of DEX. (a) In the presence of DEX alone (5.0% or 16.7%), F-actin (final 23.3 μM) was mixed with the buffer used to make stock fragmin (upper) or fragmin itself (bottom), respectively. The high concentrations of DEX and/or F-actin are meant to simulate the interior of the DEX-rich phase droplets. (b) As a control, F-actin (final 23.3 μM) was mixed with fragmin in the bulk solution, i.e., without DEX. In (a) and (b), since fragmin was found to be uniformly distributed in the PEG/DEX binary solution, as shown in Fig. 1(b), the final concentration of added fragmin remained at 7.0 μM. Fluorescence images of F-actin were acquired 5 min after the mixing. (c) Fraction of F-actin surviving with bundling/aggregation even after the addition of fragmin. For each condition in the presence of different concentrations of DEX (0%, 5.0%, and 16.7%) and in the presence (+) or absence (−) of fragmin, the ratio of the remaining fraction was obtained by dividing the sum of the fluorescence intensities of F-actins forming bundles or aggregates by the total fluorescence intensity from the entire field of view for the images as in (a) or (b). The results shown here are based on two independent experiments at each fixed condition.

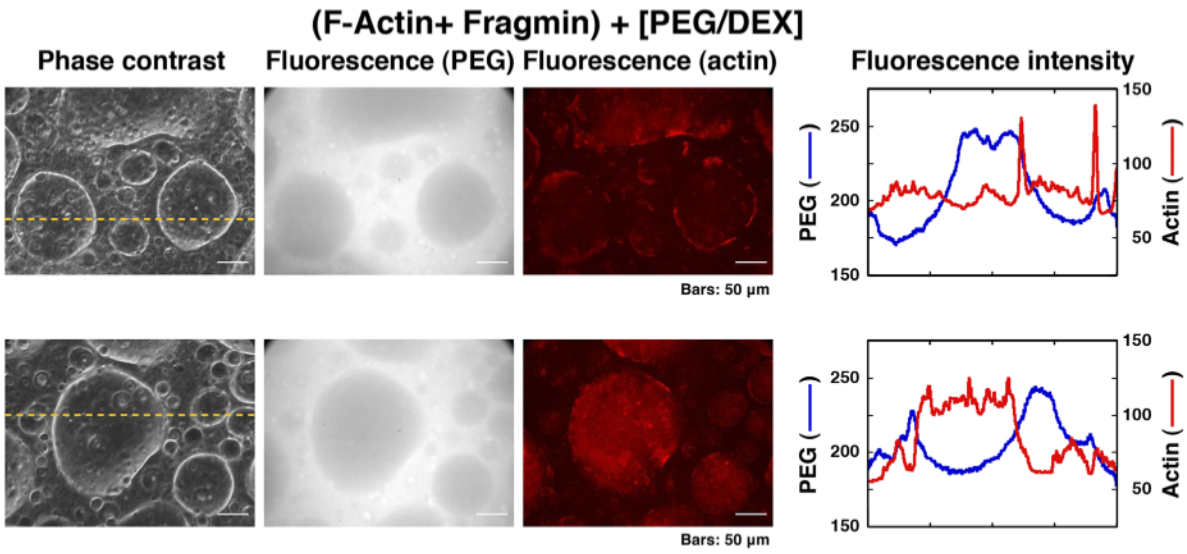

FIG. 3. Effect of LLPS on the distribution of actin that had been initially depolymerized by fragmin. From left to right, the phase contrast image, fluorescence image of PEG, and fluorescence image of actin are shown. The right graph shows the relative fluorescence intensities (arbitrary unit) of PEG (blue line) and actin (red line) obtained corresponding to the yellow broken line in the field of view in the phase contrast images. F-actin was initially mixed with the same molar amount of fragmin and complete depolymerization was confirmed from the fact that the fluorescence from actin was uniform throughout the sample, and this mixture was then added to the PEG/DEX binary solution. Two sets of examples are shown as typical results. The images were obtained soon after the solutions were mixed.

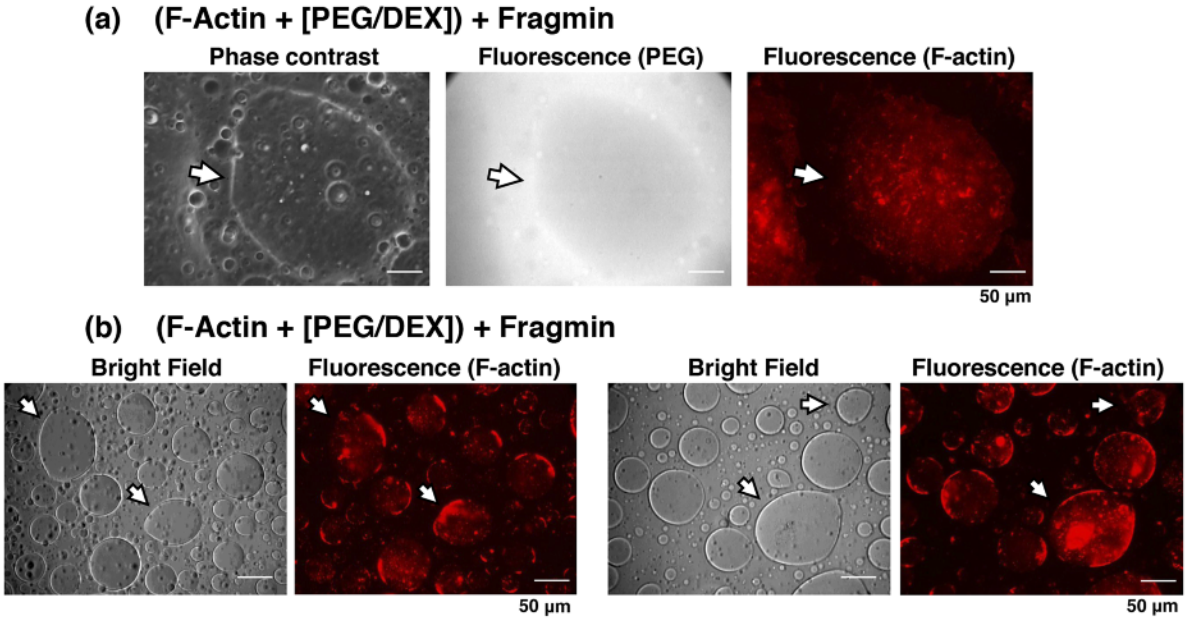

FIG. 4. The effect of fragmin on the morphology of DEX-rich phase droplets entrapping F-actin. [(a) and (b)] Non-spherical droplets (as indicated by arrows) were observed even in the presence of fragmin. In (a), phase contrast (left) and fluorescence images of the PEG-rich phase (center) or F-actin (right) are shown. In (b), bright field (left) and fluorescence images of F-actin (right) are shown. This observation was performed under condition II (see Sec. II C). The final concentrations of actin and fragmin were 6.0 and 9.0 μM [(b), left], or 9.0 and 9.0 μM, respectively [(b), right]. In all cases, images were obtained several minutes after the samples were prepared. Scale bars show 50 μm. It is noted that non-spherical droplets also appeared in Figs. 1 and 3.

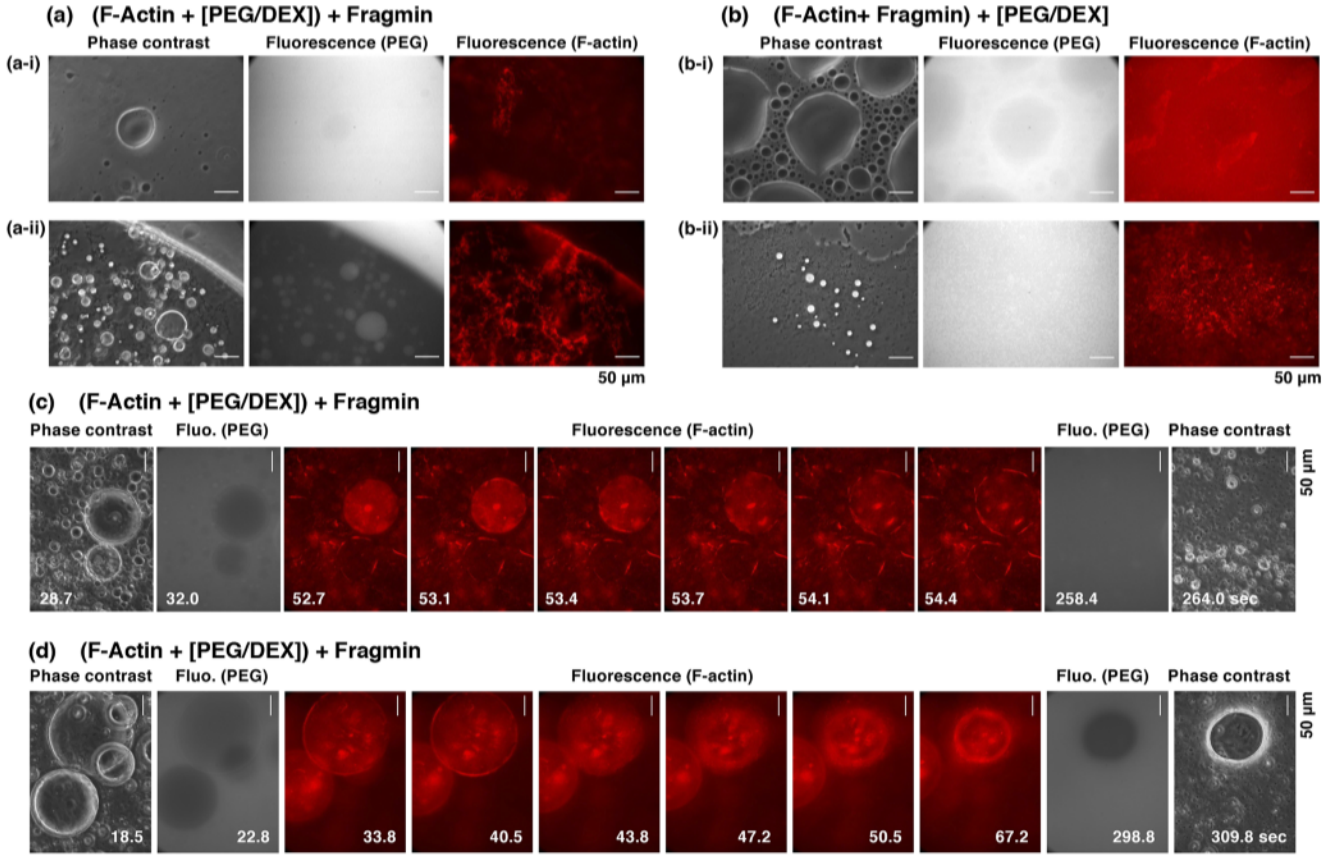

FIG. 5. Effect of fragmin on cell-sized droplets generated in a PEG/DEX binary solution in the presence of actin. (a) A PEG/DEX binary solution containing F-actin was mixed with fragmin and left for 24 h; images were then obtained. (b) F-actin that had been pre-mixed with fragmin was added to a PEG/DEX binary solution and left for 24 h; images were then obtained. In (a) and (b), two sets of examples are shown. In each panel, phase contrast (left) and fluorescence images of the PEG-rich phase (center) or F-actin (right) are shown. (c) and (d) Sequences of images showing the disappearance of a DEX-rich phase droplet (c), or a change in the interface of a DEX-rich phase droplet so as to reduce the droplet size (d), in the presence of F-actin and fragmin. The microscopy conditions are indicated above each panel, and the elapsed time (seconds) from the start of recording is indicated in each panel. For (c) and (d), see also Figs. 6 and 7, respectively. The experimental conditions are the same as in (a). Scale bars show 50 μm.

To induce this change in the distribution of F-actin to occur within tens of minutes instead of several hours, an excess molar amount of fragmin against actin was required (e.g., 3.0 μM of F-actin and 18.0 μM of fragmin) [Fig. 1(e)].

Since the volume ratio of the PEG and DEX phases in the binary solutions is approximately 7:3, inside the DEX-rich phase droplets, the concentrations of F-actins, as well as DEX, are thought to be increased up to 3.3-fold. In contrast, as shown in Fig. 1(b), the distribution of fragmin was uniform. Under conditions that simulate the interior of DEX-rich phase droplets, the actin-severing and -depolymerizing activities of fragmin were suppressed (Fig. 2).

B. Distribution of actin that was depolymerized by fragmin

Fragmin or gelsolin continues capping the barbed-end of actin even after the depolymerization they cause. Thus, the release of actin from the barbed-end capping by fragmin or gelsolin to induce the reverse reaction, i.e., actin re-polymerization, is a complex process. There are two known ways to produce such liberation: (1) binding of phosphoinositides, phospholipids with multiple negative charges, to gelsolin superfamily proteins, along with the removal of Ca^{2+}, which is essential for their activity,[32,33] and (2) simultaneously adding different types of actin-binding proteins with polymerization-promoting activity.[34]

In this study, to examine whether the environment in which LLPS occurs enables actin to polymerize even in the presence of fragmin, actin that was depolymerized by fragmin in advance was added to the PEG/DEX binary solution. Within several minutes, F-actins, which were considered to be the product of the reverse polymerization reaction, were observed in the DEX-rich phase droplets (Fig. 3). These F-actins frequently formed aggregations or bundles, as in the absence of fragmin. The results obtained did not depend on whether fluorescent labeling consisted of the addition of

rhodamine–phalloidin or covalent modification with Alexa (Fig. S3). This restricted localization was temporary. After that, F-actins localized in the DEX-rich phase droplets gradually decreased, while actin in the PEG phase increased (Fig. S4). The amount of actin that was temporarily localized in the DEX-rich phase depends on the mixing ratio of F-actin and fragmin.

If we consider all of the results, in the PEG/DEX binary solution in which LLPS is occurring, actin polymerization seems to be helped by its concentration in DEX-rich phase droplets, regardless of the mixing order among actin, its depolymerizing factor, and macromolecules. This is consistent with the fact that the threshold concentration of salts required to polymerize actin is reduced in the PEG/DEX binary solution, resulting in the much easier formation of F-actin.[22]

C. Effect of actin on the morphology of droplets

When F-actins are localized in DEX-rich phase droplets, their morphology often deviates from spherical shape.[22] There are two major reasons for this observation. First, F-actins localized within a droplet can form large aggregates or long bundles, and work to push the boundary of the droplet outward from the inside, or make the interface of the droplet flat like the surface of a polygon (Fig. 4). Second, when F-actins localize beneath the boundary of the droplets, although contact among the droplets occurs, a post-fusion change in morphology to a spherical shape does not occur, resulting in the formation of a peanut-like shape. Because F-actins localized in the DEX-rich phase droplets remained without depolymerization even with the addition of fragmin, non-spherical droplets were still found (Fig. 4).

Droplets became unstable after being left for a long time, and the boundary between the PEG- and DEX-rich phases was disrupted and became unclear (Figs. 5–7). In Figs. 5(a-i) and 5(b), the intensity and contrast of images of fluorescence-labeled PEG (each center) are enhanced as much as possible. Otherwise, the difference in PEG concentrations in the binary solution was indistinguishable.

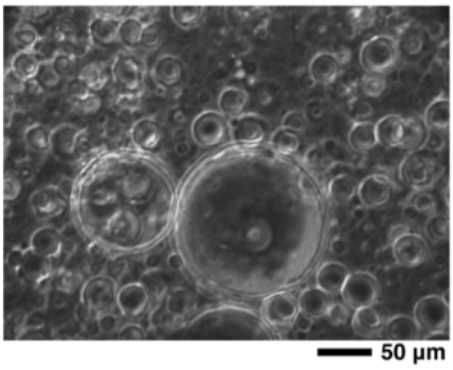

FIG. 6. Video movie shows disappearance of a DEX-rich phase droplet, as observed in the presence of F-actin and fragmin. This is the image obtained at 30 frames/s, from 25 to 272 s after the start of recording and is shown in real-time. The details are described in the caption of Fig. 5. Multimedia view: https://doi.org/10.1063/5.0055460.1

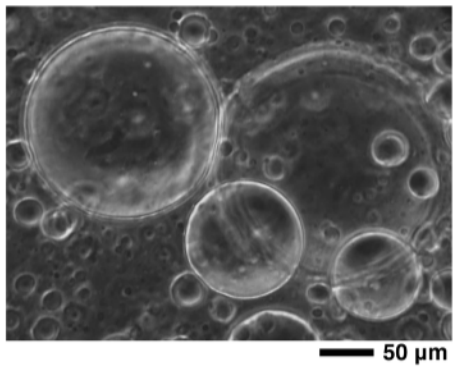

FIG. 7. Video movie shows change in the interface of a DEX-rich phase droplet, as observed in the presence of F-actin and fragmin. This is the image obtained at 30 frames/s, from 14 to 310 s after the start of recording and is shown in real-time. The details are described in the caption of Fig. 5. Multimedia view: https://doi.org/10.1063/5.0055460.2

Therefore, the droplet-like regions seen in phase contrast images are not always the DEX-rich phase that is normally caused by LLPS. These occasionally stick to a glass substrate, such as the wall of the sample [Fig. 5(b-i)]. In addition, many small droplets of one phase were found inside the droplets of the other phase [Fig. 5(a-ii)]. These PEG-rich phase droplets are apparently trapped in the small gaps within meshes formed by many short F-actins. This destabilization of cell-sized droplets was observed only when both actin and fragmin were added.

IV. DISCUSSION

A. Actin regulation by LLPS

We have reported that, in the presence of DEX-rich microdroplets surrounded by the PEG-rich solution, F-actin is condensed into DEX-rich droplets, whereas G-actin is uniformly distributed throughout both the DEX and PEG-rich phases. These results are attributable to the depletion force of the inert macromolecules DEX and PEG.[22,35,36]

In this report, we demonstrated that F-actins localized in DEX-rich phase droplets are protected from a depolymerizing factor, fragmin, and the actins that were initially severed and depolymerized by fragmin are temporarily localized in DEX-rich phase droplets, which is attributable to their re-polymerization.

Under the physicochemical conditions in the DEX-rich phase droplet, fragmin does not depolymerize bundles/aggregates of F-actins promptly. Thus, the experimental observation of the accumulation of F-actins inside droplets and of the subsequent bundling or aggregation in the DEX-rich environment indicates that the depletion force of the coexisting polymers, PEG and DEX, plays an important role. As a result of bundling and/or aggregation, fragmin may be blocked from accessing F-actins inside those structures due to the narrow spacing. Alternatively, even if F-actins are severed, the shortened ones may remain in aggregates or bundles. An F-actin bundle can also be induced by a huge inert polymer,

methylcellulose and can be accessed by myosin motor molecules and slide and deform its shape and length.[37,38] Therefore, the arrangement of F-actins that is determined by the depletion force of surrounding macromolecules is important for regulating the effective activities of related enzymes. The depletion force of PEG and DEX also causes the positive feedback between the restricted localization of actin in the DEX-rich phase droplets as a result of polymerization and the facilitation of polymerization by accumulation due to restricted localization.[22] On the other hand, no apparent effect on the fragmin distribution was observed. These observed phenomena should contribute to the re-polymerization of actin in the presence of fragmin. In any case, the LLPS could be a novel mechanism for the recovery of actin cytoskeleton from the depolymerization state caused by a gelsolin superfamily protein.[32–34]

Importantly, actin does not specifically interact with either PEG or DEX. Therefore, a simple biochemical mechanism, such as the key-lock reaction seen between an enzyme and its substrate, cannot be the basis for the actin behaviors found in this study. Instead, the environment where micro-segregation of a polymer solution occurs is important for these findings. The differences between the macromolecules used, i.e., PEG is a flexible linear polymer, while DEX is a semiflexible branched polymer, are responsible for the LLPS caused in the binary solution. The depletion force or the entropic effect of the crowding polymer conformation, resulting from the coexistence of these two macromolecules, should be a driving force for the characteristic behavior of actin in a solution with water/water micro-droplets found in this study.[21,39,40]

The cell interior is a space where various macromolecules including the actin cytoskeleton itself exist at extremely high concentrations. Therefore, it is quite possible that the changes in the behavior of actin observed in this study are present in living cells. To date, when researchers have considered the mechanism that regulates the cytoskeleton, they have focused on the regulatory factors that directly or indirectly bind to the cytoskeleton and/or the expression of genes that code them.[41–43] In the future, mechanisms based on phase separation should also be considered.

B. Effect of actin on cell-sized droplet formation

The change in the shape of cell-sized DEX-rich phase droplets caused by the localization of F-actin, but not of long double-stranded DNA,[22,25] is attributable to the dynamics and stiffness of the actin cytoskeleton. When the concentration of F-actin within a droplet increases, it tends to form bundles and localize at the interface of the droplet. When the linear rigid structure exists at the interface, the shape of the droplet will be easily deformed from the spherical shape. If F-actin is responsible for the unique morphology of the droplets, it is possible that the factors involved in suppressive regulation of F-actin, such as depolymerization, could restore the droplets to a spherical shape. Although the effects of fragmin, a representative of such suppressive regulatory factors, were examined, fragmin hardly made the droplets spherical because F-actins in the DEX-rich phase remained in the droplets as mentioned above. This finding shows that the effect of F-actin on droplet morphology is robust to its suppressive regulation.

On the other hand, droplet formation and phase separation became unstable, and the boundaries between the PEG- and DEX-rich phases were disturbed and obscured. The coexistence of F-actins in a PEG/DEX binary solution may be able to alter the conditions of phase separation suitable for micro-segregation, e.g., depending on their length or distribution. If so, since fragmin changes both the length and distribution of actin in the binary solution, this may result in the observed changes in the stability or size of droplets.

Since a large amount of actin is expressed in every eukaryotic cell, in both the cytoplasm and nucleus,[44–47] it is possible that such actins are involved in the maintenance of membraneless microcompartments formed inside cells. A previous study using DNA showed that long double-stranded DNA is localized only in cell-sized DEX-rich phase droplets in the binary solution and DEX-rich phase droplets can be manipulated by manipulating this DNA.[48] Similarly, actin may be useful as a tool for manipulating micro-segregation in *in vivo* or artificial systems.

V. CONCLUSION

The polymerization/depolymerization dynamics of the actin cytoskeleton, which is one of the most important keys to inner cellular regulation, may be significantly altered both qualitatively and quantitatively in an environment where biological macromolecules are congested and thus LLPS occurs.

In addition, the present results suggest that not only does LLPS affect the dynamics of the actin cytoskeleton, but also the behavior of the cytoskeleton can affect micro-segregation.

An important role of the actin cytoskeleton, other than in the arrangement of intracellular mechanical structures or the treadmill reaction, which are based on actin polymerization/depolymerization dynamics, is the generation of movement or force in collaboration with the molecular motor myosin. Thus, it should be worthwhile to investigate the relationship between the micro-segregation of aqueous polymer solution and the dynamical structure and function of the cooperative system of actin and myosin.

SUPPLEMENTARY MATERIAL

See the supplementary material for Figs. S1–S4.

AUTHORS' CONTRIBUTIONS

T.W., H.S., M.H., K. Tsumoto, K. Takiguchi, and K.Y. conceived this project. T.W., H.S., and M.H. conducted experiments. M.H., K. Tsumoto, and K. Takiguchi wrote and summarized the manuscript with feedback from the coauthors. K.Y. provided supervision.

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The authors declare no conflict of interest.

DATA AVAILABILITY

The data that support the findings of this study are available within the article and the supplementary material.

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SUPPLEMENTARY MATERIAL:

Polymerization/depolymerization of actin cooperates with the morphology and stability of cell-sized droplets generated in a polymer solution under a depletion effect

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Supplementary Figures

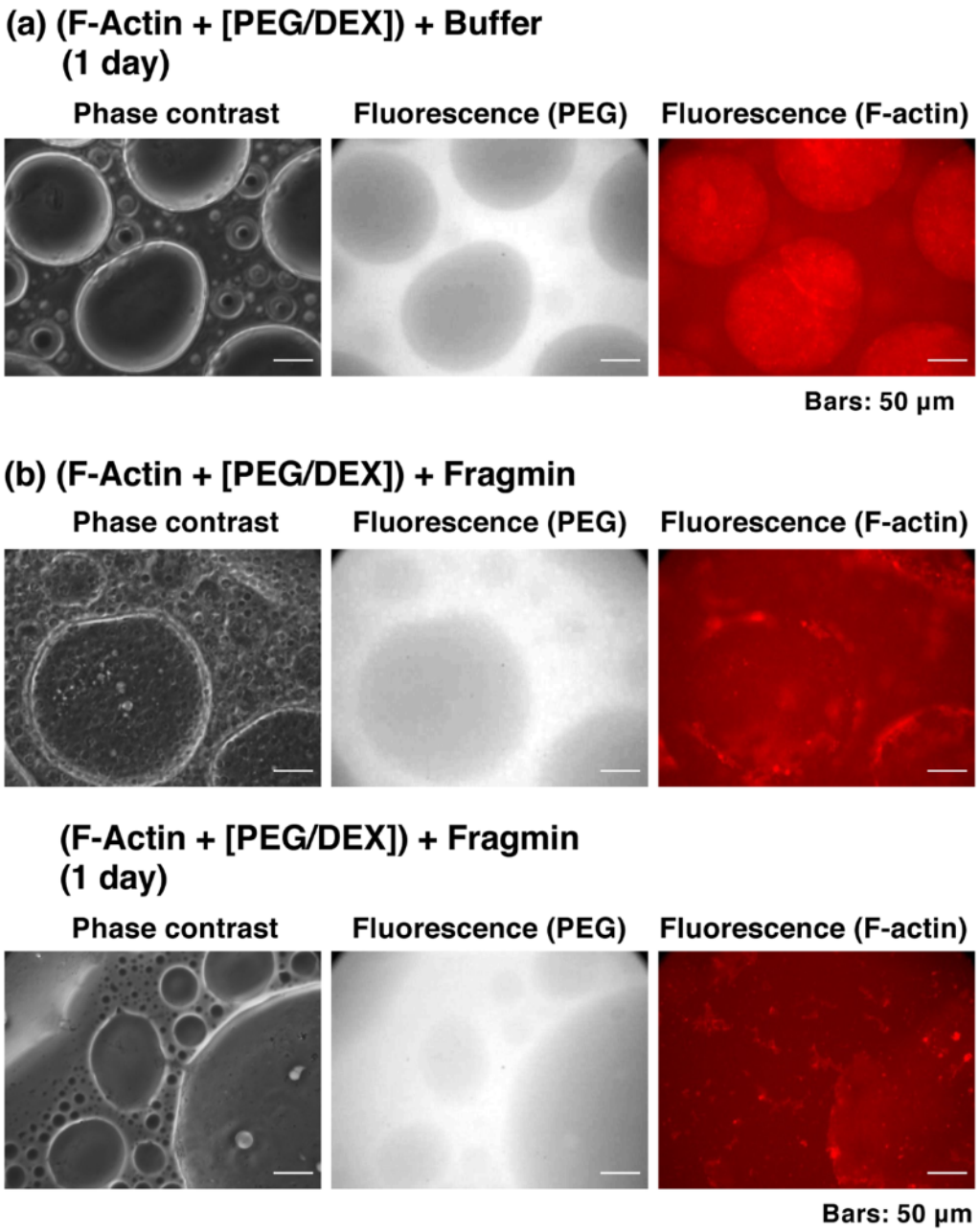

Figure S1. Actin distribution in the PEG/DEX binary solution after addition of fragmin. The experimental conditions in (a) and (b) were the same as in Fig. 1c and 1d, respectively, except labeling method of actin. Actin was labeled covalently with Alexa 546, instead with the addition of rhodamine-phalloidin.

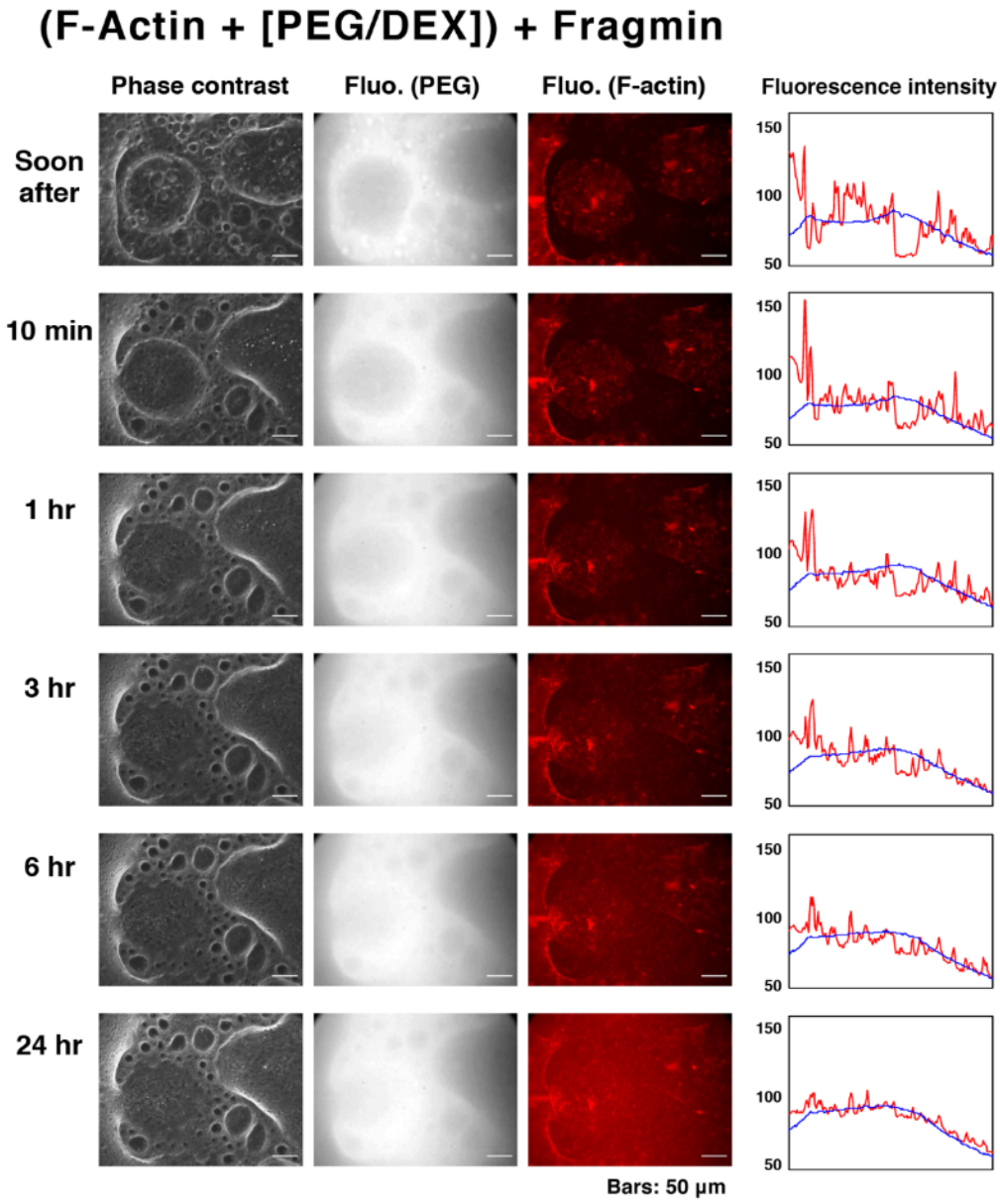

Figure S2. The time-course of change observed after addition of fragmin to an F-actin-containing PEG/DEX binary solution. The elapsed time after the mixing is indicated on each panel. From left to right, phase contrast image, fluorescence image of PEG, and fluorescence image of actin. The right graph shows the relative fluorescence intensities (arbitrary unit) of PEG (blue line) and actin (red line) plotted along the pixels (based on the images acquired by twice compressing the corresponding original images of 400 pixels/400 µm) of the full width of the equator of each image. The experimental conditions were the same as in Fig. 1d.

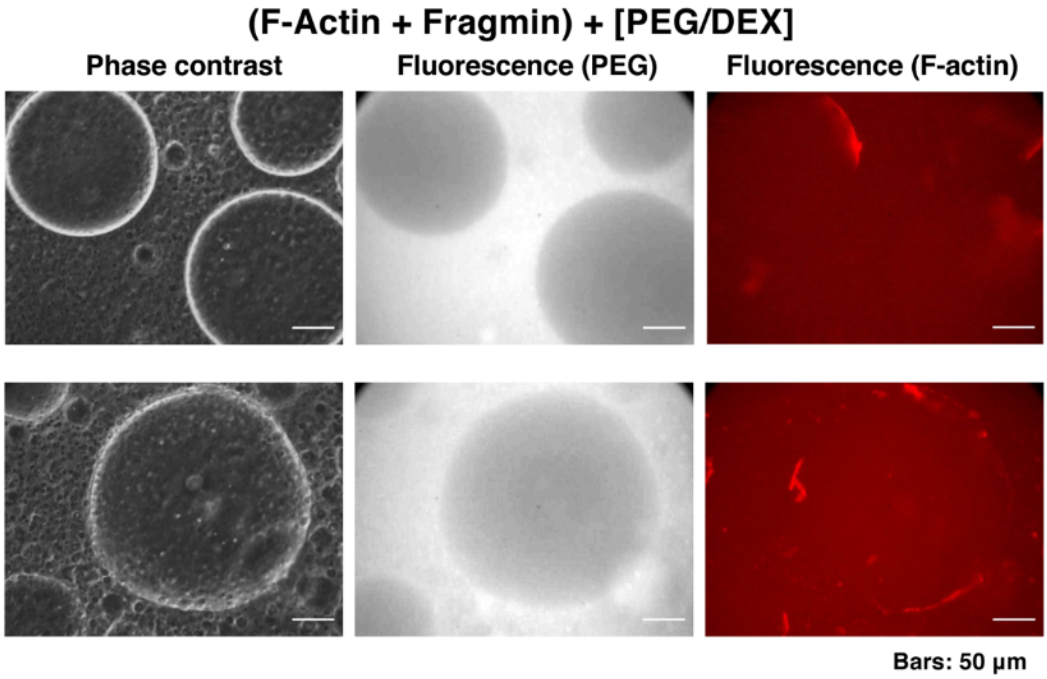

Figure S3. Effect of LLPS on the distribution of actin that had been initially depolymerized by fragmin. The experimental conditions were the same as in Fig. 3, except labeling method of actin. Actin was labeled covalently with Alexa 546, instead with the addition of rhodamine-phalloidin. Two sets of examples are shown.

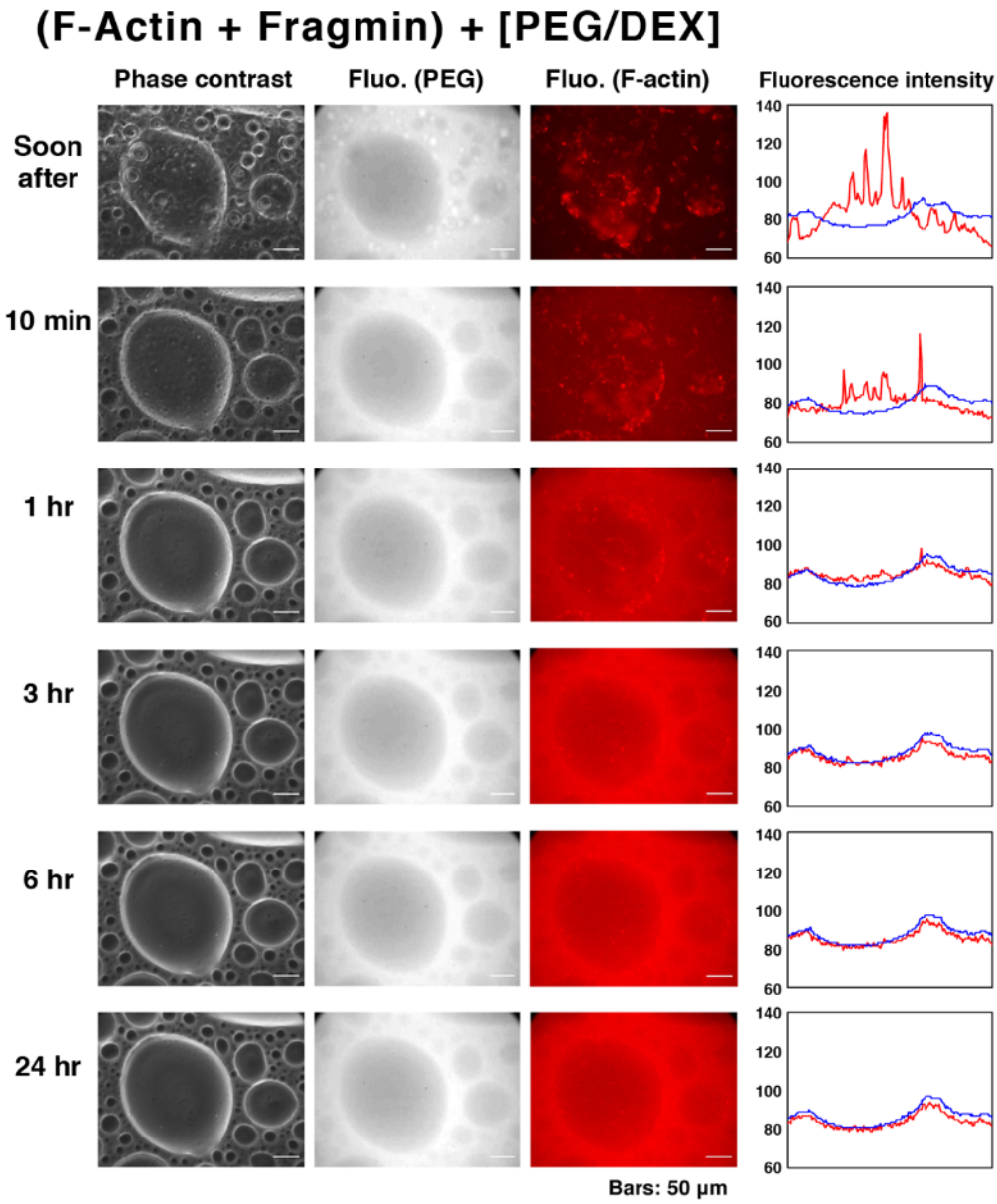

Figure S4. The time-course of changes observed after addition of a mixture of F-actin and fragmin to a PEG/DEX binary solution. The elapsed time after the mixing is indicated on each panel. From left to right, phase contrast image, fluorescence image of PEG, and fluorescence image of actin. The right graph shows the relative fluorescence intensities (arbitrary unit) of PEG (blue line) and actin (red line) plotted along the pixels (based on the images acquired by twice compressing the corresponding original images of 400 pixels/400 μm) of the full width of the equator of each image. The experimental conditions were the same as in Fig. 3.