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Microscopic Temperature Control Reveals Cooperative Regulation of Actin–Myosin Interaction by Drebrin E

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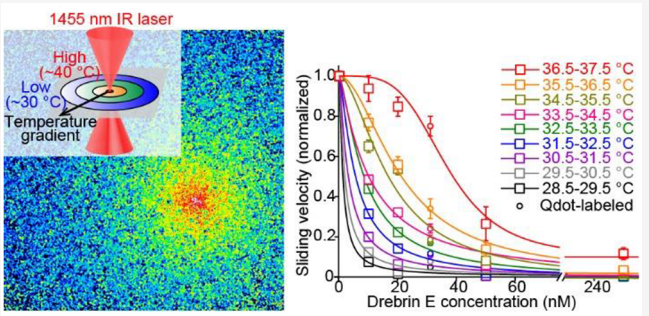
ABSTRACT: Drebrin E is a regulatory protein of intracellular force produced by actomyosin complexes, that is, myosin molecular motors interacting with actin filaments. The expression level of drebrin E in nerve cells decreases as the animal grows, suggesting its pivotal but unclarified role in neuronal development. Here, by applying the microscopic heat pulse method to actomyosin motility assay, the regulatory mechanism is examined from the room temperature up to 37 °C without a thermal denaturing of proteins. We show that the inhibition of actomyosin motility by drebrin E is eliminated immediately and reversibly during heating and depends on drebrin E concentration. The direct observation of quantum dot-labeled drebrin E implies its stable binding to actin filaments during the heat-induced sliding. Our results suggest that drebrin E allosterically modifies the actin filament structure to regulate cooperatively the actomyosin activity at the maintained in vivo body temperature.



KEYWORDS: *actin-binding protein, cytoskeleton, heat pulse, molecular motor, motility assay, nanofilament regulation, temperature imaging*

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Actomyosin force generation, the crucial event governing cell motility and morphology at the nanoscale, is coupled with the conversion of the chemical energy of adenosine triphosphate (ATP) into work by enzymatic activity. The chemo-mechanical conversion varies spatiotemporally in each cell in dependence on its environment. This process is precisely regulated by modulating the interaction between an actin filament, which comprises linearly polymerized monomers with a diameter of ∼7 nm, and myosin molecules via the actin-binding proteins, such as drebrin in the case of nerve cells.[1]

Drebrin is an essential regulator of the neurite outgrowth.[2−4] It is suggested that drebrin regulates various properties of an actin cytoskeleton by binding to actin filaments in a competitive manner with other actin-binding proteins, such as α-actinin, tropomyosin, and fascin in nerve cells.[5,6] It is classified into two isoforms, embryo (E) and adult (A), and both isoforms are side-binding proteins of the actin filament. The domination of drebrin E at early stages is followed by its minor expression level at later stages during animal development.[7]

Reconstituted systems that use purified proteins have revealed that drebrin can inhibit actomyosin interaction. For example, drebrin binding to an actin filament causes the decrease of sliding velocity of actin filaments driven by myosin II[8] or myosin V molecules[9] attached to a glass substrate. A suppression of force generated by myosin V was also demonstrated in a single molecule assay.[10] These results have suggested that drebrin modulates the mechanochemical properties of myosin by a competitive binding to actin filaments. However, a correlation between the drebrin concentration and the actomyosin activity remains unclear. While the dissociation constant (K_d) between drebrin E and actin filaments is reported to be 120 nM,[5] this value seems inconsistent with the results on the reconstituted systems where actomyosin motility was significantly suppressed in the presence of 10 nM of drebrin E.[8,10] Furthermore, experiments have been limited to temperatures much lower than the physiological temperature, because thermal damage on purified proteins, especially molecular motors such as myosin, had to be avoided.[11]

In the current study, we applied the optically controlled microscopic heat pulse method to the sliding assay of actomyosin, namely, the in vitro motility assay. Relatively fast heating in this method enables an examination of the

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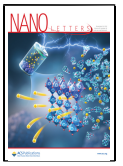

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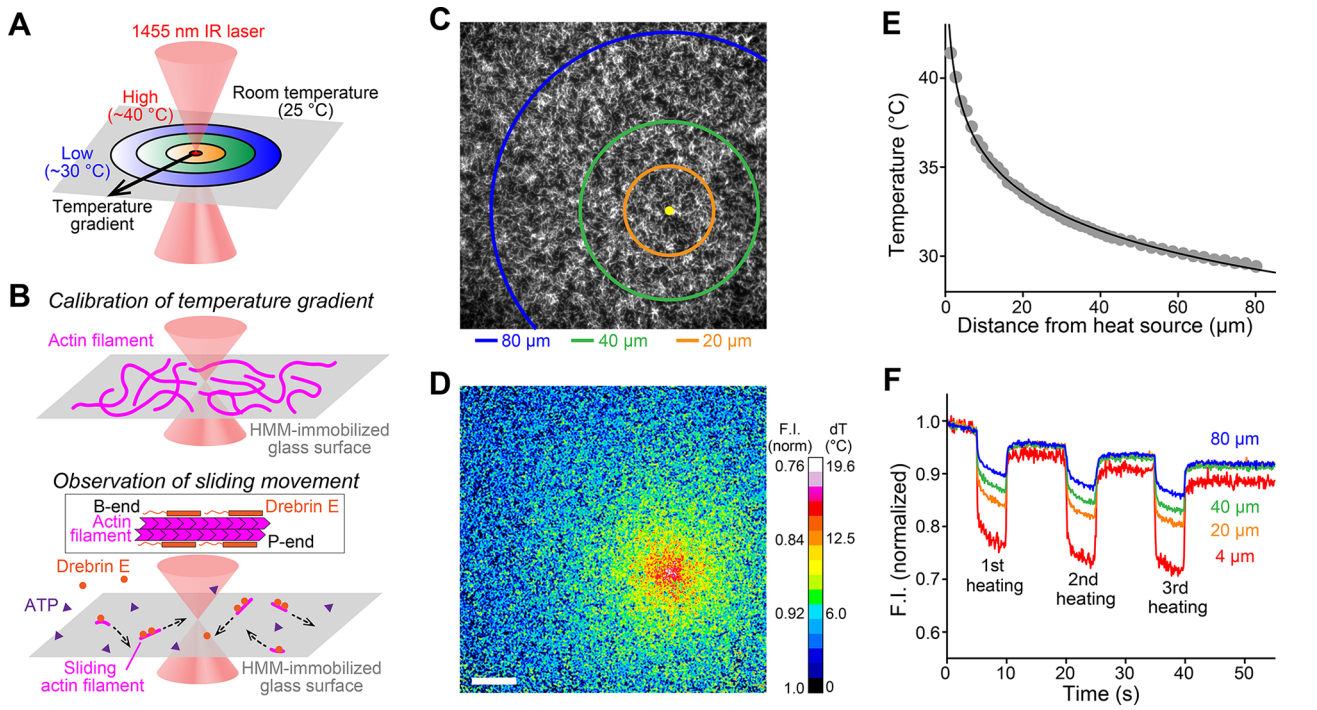

Figure 1. Optically created microscopic heat pulse to examine the actin−myosin interaction at the physiological temperature. (A) Schematic illustration of the microscopic heat pulse. Concentric temperature gradient is formed by a 1455 nm IR laser light focused on aqueous medium. (B) Schematic illustrations of flow cells for calibration of temperature gradient (top) and for measurements of sliding velocity of actin filaments (bottom). The calibration of temperature gradient was performed in the absence of ATP using relatively long actin filaments in high density attached to heavy meromyosin (HMM) molecules on the glass surface. Sliding velocity was determined in the presence of ATP using relatively short, ∼1−5 μm long actin filaments and various concentrations of drebrin E. Schematic illustration of the bottom, inset, for the drebrin-actin interaction is based on previous studies reporting that one drebrin molecule is bound to five actin filament subunits[5] and that the N-terminal globular domain and C-terminal loop structure of drebrin are directed to pointed (P-) and barbed (B-) ends of an actin filament, respectively.[15] (C) Representative fluorescence image of actin filaments labeled with rhodamine−phalloidin during the calibration of temperature gradient. A filled yellow circle indicates the position of the heat source. Colored circles represent, respectively, the points 20, 40, and 80 μm away from the heat source. (D) Temperature map during heating determined from the fluorescence intensity (F.I.) of rhodamine normalized to that before heating (scale bar, 20 μm). Corresponding video can be viewed in Movie S1. (E) The temperature around the heat source determined from the temperature map as in (D), and the thermosensitivity of rhodamine dye (Figure S1) as a function of the distance from the heat source approximated by the function $y = -3.1 \ln(x/x_0) + 42.9$. Closed circles and a solid curve are the data experimentally obtained and the fitting according to the above function, respectively. In order to have a unitless quantity when taking the log, we set $x_0 = 1$ (μm). (F) Time course of the mean fluorescence intensities at 4, 20, 40, and 80 μm away from the heat source normalized to that at 0 s.

actomyosin activity up to the physiological temperature without a thermal denaturation of proteins.[11,12] We successfully determined the thermal sensitivity of the inhibitory effects by various concentrations of drebrin E with the single-filament resolution. On the basis of our results, we propose that the inhibition of actomyosin activity by drebrin E is not a simple competitive binding to actin filaments with myosin but involves a cooperative modification of actin filament structure. The physiological significance of the thermal sensitivity is also discussed.

MICROSCOPIC TEMPERATURE CONTROL BY OPTICAL MICROHEATING

Microscopic heat pulses were applied by focusing a 1455 nm infrared (IR) laser light in the field of view of the fluorescence microscope where actin filaments were observed (Figures 1, S1, and S2; Movie S1).[12−14] A concentric temperature gradient is created by an IR laser in the area within several tens of micrometers from the focal point. The switching (on/off) of the temperature gradient was controlled with the 0.1 s resolution by the IR laser light shutter (Figure 1F). The highest temperature over 40 °C (room temperature 25 °C + the temperature rise caused by the IR laser light) was obtained at the focal point, while the border temperature of the field of view was ∼30 °C. The area within ca. 10 μm from the heat source (focal point) was suitable for the measurements at the physiological temperatures (35.8, 36.6, and 37.9 °C at 10, 7.5, and 5 μm from the heat source, respectively).

HEAT-INDUCED REDUCTION OF DREBRIN E-MEDIATED INHIBITION OF ACTIN FILAMENT SLIDING

The effect of elevated temperature on the inhibition of actomyosin interaction by drebrin E was examined using the microscopic heat pulse and the in vitro motility assay (Figure 2). The in vitro motility assay that uses purified myosins and actin filaments has been a useful tool to examine the actomyosin motile activity, where it can be quantitatively visualized as sliding motions of individual actin filaments under the fluorescence microscope (Figure 2A).[16] For example, the enhanced activity of actomyosin at elevated temperature is manifested as the accelerated sliding velocity during heating[11,17] (Figure 2B, "0 nM"; Movie S2).

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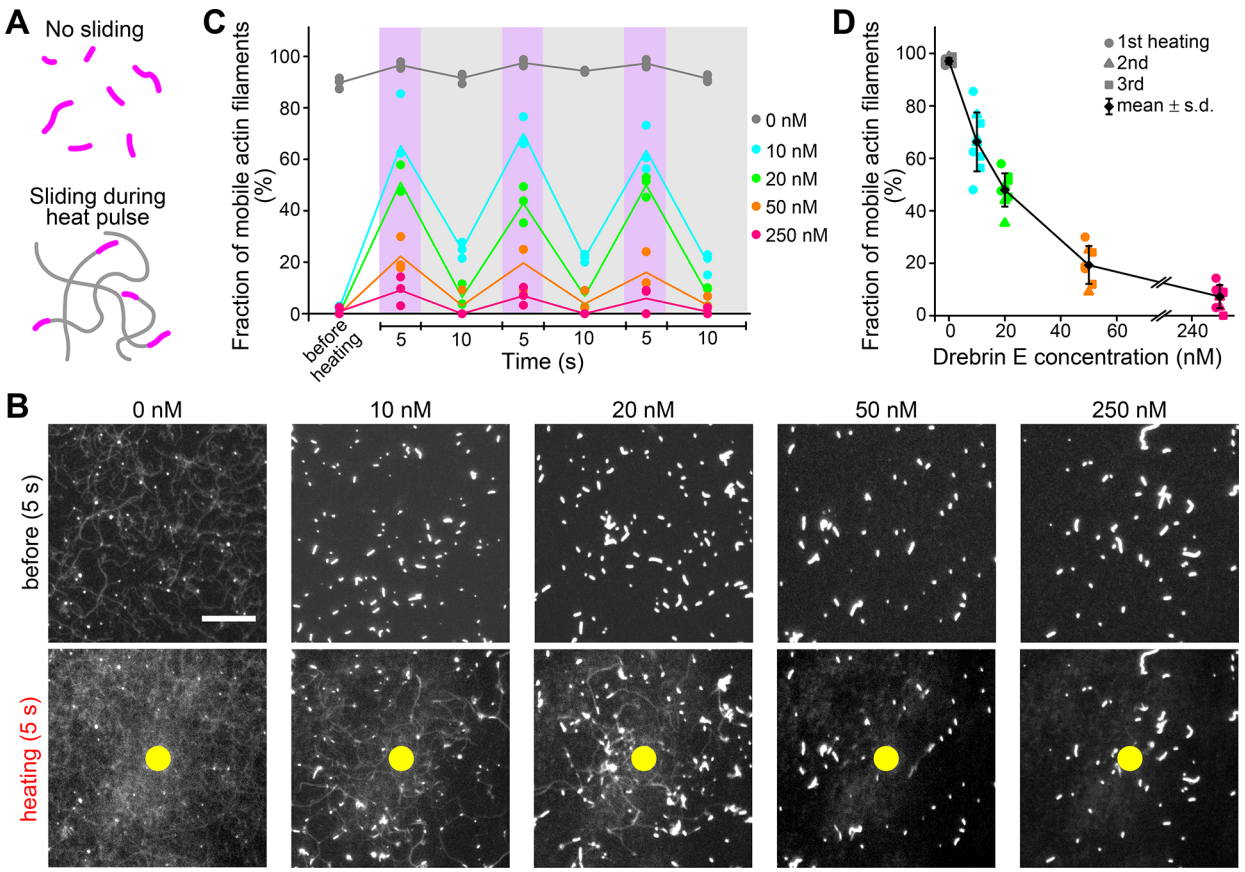

Figure 2. Heat-induced activation of the actin filaments sliding inhibited by drebrin E. (A) Schematic illustration showing how stationary (above) and sliding (below) actin filaments were observed in overlaid images. (above) Actin filaments are observed as bright objects remaining in the same locations before and during a heat pulse. (below) The trajectories of sliding actin filaments with a lower fluorescence intensity are formed (illustrated by gray lines). Hence, initially stationary filaments that slide during heating are manifested as individual curves composed of a short bright segment followed by a long faint tail. (B) Fluorescence images before (above) and during (below) heating in the presence of various concentrations of drebrin E. Maximum intensities of 50 frames were overlaid for each period. Filled yellow circles indicate the positions of the heat source (scale bar, 20 μm). (C) Fraction of mobile actin filaments (see the Supporting Information for definitions) after three repeated heat pulses in the presence of various concentrations of drebrin E as indicated in the legend. The lines connect the means obtained in three individual experiments. Pink and gray vertical bars indicate the periods of heating and intervals between them, respectively. Heat pulses as shown in Figure 1D,E were applied. (D) Fraction of mobile actin filaments as a function of drebrin E concentration. Plots were reproduced from the data shown in (C). The line connecting the means is drawn for guiding an eye.

In the presence of drebrin E, majority of actin filaments demonstrated no sliding (defined as immobile, see Supporting Information) before heating (Figure 2B, top).[8] However, we found that the sliding (mobile filament) was initiated upon heating (Figure 2B, bottom). The switching between mobile and immobile statuses was reversible (Movies S3−S6). The sliding was observed only in actin filaments in the vicinity of the heat source, and the fraction of mobile actin filaments decreased as drebrin E concentration increased (Figure 2C,D). Note that the increased fraction of mobile filaments during the intervals between the heating cycles compared to that before the first heating (before heating) is attributed to the relaxation time required for the full recovery of inhibition (the time constant of 5.4 s in the case of 30 nM drebrin E; Figure S3). These results indicate that the actomyosin regulation by drebrin E is dependent on both temperature and drebrin E concentration.

DIRECT OBSERVATION OF DREBRIN E ATTACHED TO MOBILE ACTIN FILAMENT DURING HEATING

The repeatable and reversible heat-induced sliding by switching on/off the laser pulse could be simply explained by the thermal dependence of the drebrin E affinity to actin filaments; that is, drebrin E detaches from actin filaments due to a lowered affinity during heating, then reattaches as the affinity recovers during cooling. This possibility was examined by a cosedimentation assay (Figure S4) followed by a direct imaging of the behavior of drebrin E molecules labeled with quantum dots (Qdots) via antidrebrin E antibody (Figure 3A,B and Movies S7 and S8). Ishikawa et al. has determined the K_d of drebrin E as 120 nM by using Coomassie Brilliant Blue staining for sodium dodecyl sulfate poly(acrylamide) gel electrophoresis.[5] The same authors later commented that the estimate could be inaccurate in their method of staining, which was technically difficult to determine very low K_d.[1,18] Therefore, the K_d of drebrin E was re-evaluated by luminescence densitometry, and we determined K_d = 6 nM at 25 °C, orders of magnitude smaller than the value previously reported (Figure S4). We also found that the value is elevated

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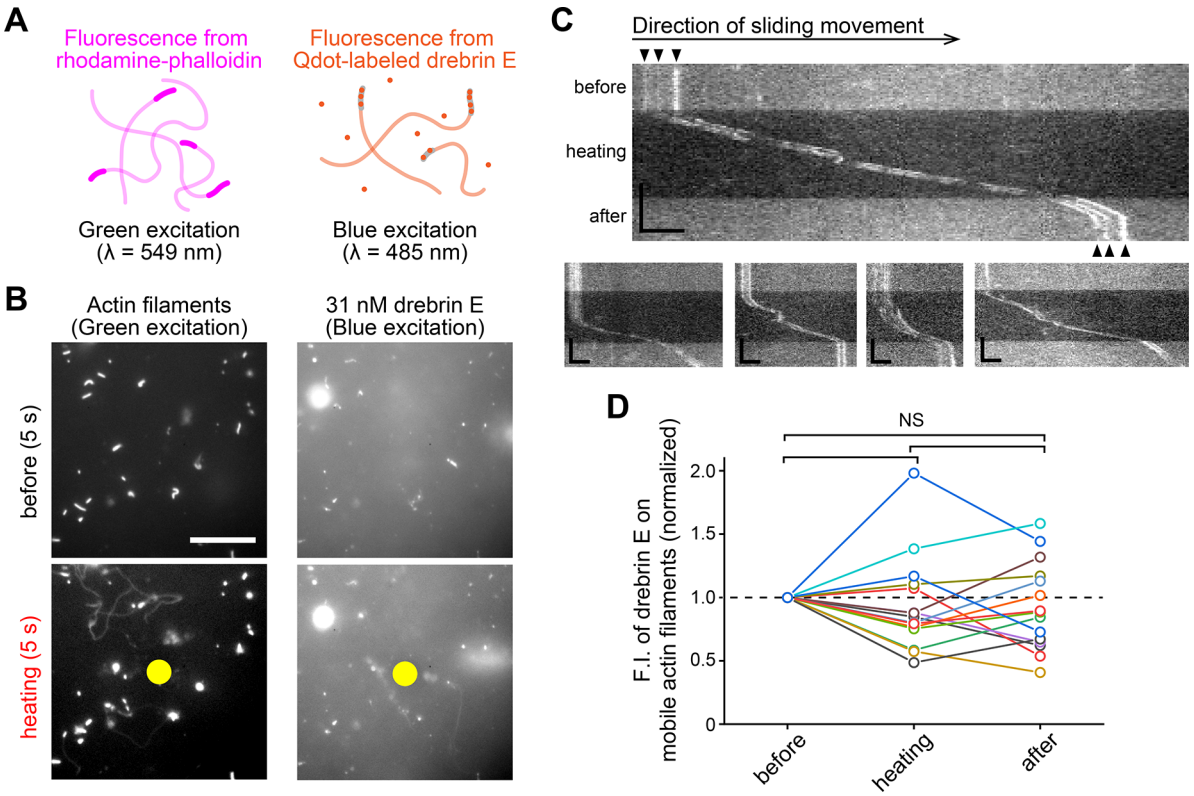

Figure 3. Direct observation of drebrin E attached to actin filaments during heating. (A) Curves composed of a short bright segment followed by a long faint tail schematically illustrate trajectories of rhodamine−phalloidin (left) and Qdot-labeled drebrin E molecules (right) on mobile actin filaments. Actin filaments and Qdots are manifested as bright objects when they remain in the same location. (B) Fluorescence images of rhodamine (left) and Qdots (right) before and during heating. Actin filaments labeled with rhodamine−phalloidin were observed at green excitation (λ_{ex} = 549 nm), whereas Qdots (λ_{ex} = 485 nm) on the actin filaments were observed in different locations in the same flow cell. Maximum intensities of 50 frames were overlaid in each period. Yellow circles indicate the positions of the heat source. The concentration of Qdot-labeled drebrin E (31 nM) is an estimation obtained as a sum of drebrin E molecules in free form and in complex with Qdots (see the Supporting Information) (scale bar, 20 μm). (C) Five representative kymographs of mobile actin filaments observed via Qdot-labeled drebrin E. Kymographs were created along the trajectories of sliding motions (illustrated as curves in (A)) for the time periods before heating (2.5 s), during heating (5 s), and after heating (2.5 s). Arrow heads in the top image indicate three Qdot spots on the mobile actin filament. Scale bars (black) shown at the bottom left of each kymograph are 2.5 s and 5 μm for vertical (time) and horizontal (displacement) axes, respectively. (D) Fluorescence intensity (F.I.) of Qdot-labeled drebrin E on mobile actin filaments before, during, and after heating. Heat pulses as shown in Figure 1D,E were applied. Scatters connected by lines of the same color represent sequential data from individual actin filaments (n = 15). F.I. values were statistically compared by a Friedman analysis of variance (p = 0.344; NS, not significant).

to 32 nM at 37 °C. Drebrin E detachment from actin filaments by the lowered affinity during heating, if it takes place, should be observed as the detachment of Qdots, resulting in a decrease in their fluorescence intensity. However, Qdots were recognizable without any changes throughout the entire period of observations before, during, and after the heating (Figure 3B and Movie S7). Multiple spots of Qdots located on individual mobile filaments remained in the same locations in all the filaments observed (Figure 3C and Movie S7). These observations were supported by a quantitative analysis of the Qdot filament trajectories (Figure 3C). Although the fluorescence intensity of Qdot either increased or decreased during and after the heating in individual filaments, these changes appeared at random. There was no significant decrease or increase of the population during the observation (Figure 3D). We also confirmed that the inhibitory effect of drebrin E, which had been preincubated at 45 °C for 1 min, remained statistically the same to that of nontreated drebrin E (Figure S5). To summarize, drebrin E appeared to remain attached to the actin filament during the heat-induced sliding, no additional binding of drebrin E was recognized after heating, and drebrin E was not thermally denatured by heating.

DEPENDENCE OF DREBRIN E INHIBITORY EFFECT ON TEMPERATURE AND CONCENTRATION

Finally, the sliding velocity was determined at various temperatures and drebrin E concentrations (Figures 4A and S6). The sliding velocity of actin filaments increased monotonically with temperature rise at all the tested drebrin E concentrations (Figure 4B). Furthermore, the temperature behavior was heavily dependent on the drebrin E concentration. At 28.5−29.5 °C, the sliding was almost suppressed in the presence of 10 nM of drebrin E (Figure 4B). However, the velocity in the presence of 10 nM of drebrin E was remarkably increased at an elevated temperature and reached a value comparable to that at 0 nM and 36.5−37.5 °C. This trend is similar but less significant in the presence of 50 and 250 nM of drebrin E, where the sliding velocity remains low even at 36.5−37.5 °C.

Then, the inhibitory effect as a function of drebrin E concentration was examined by replotting the data in each

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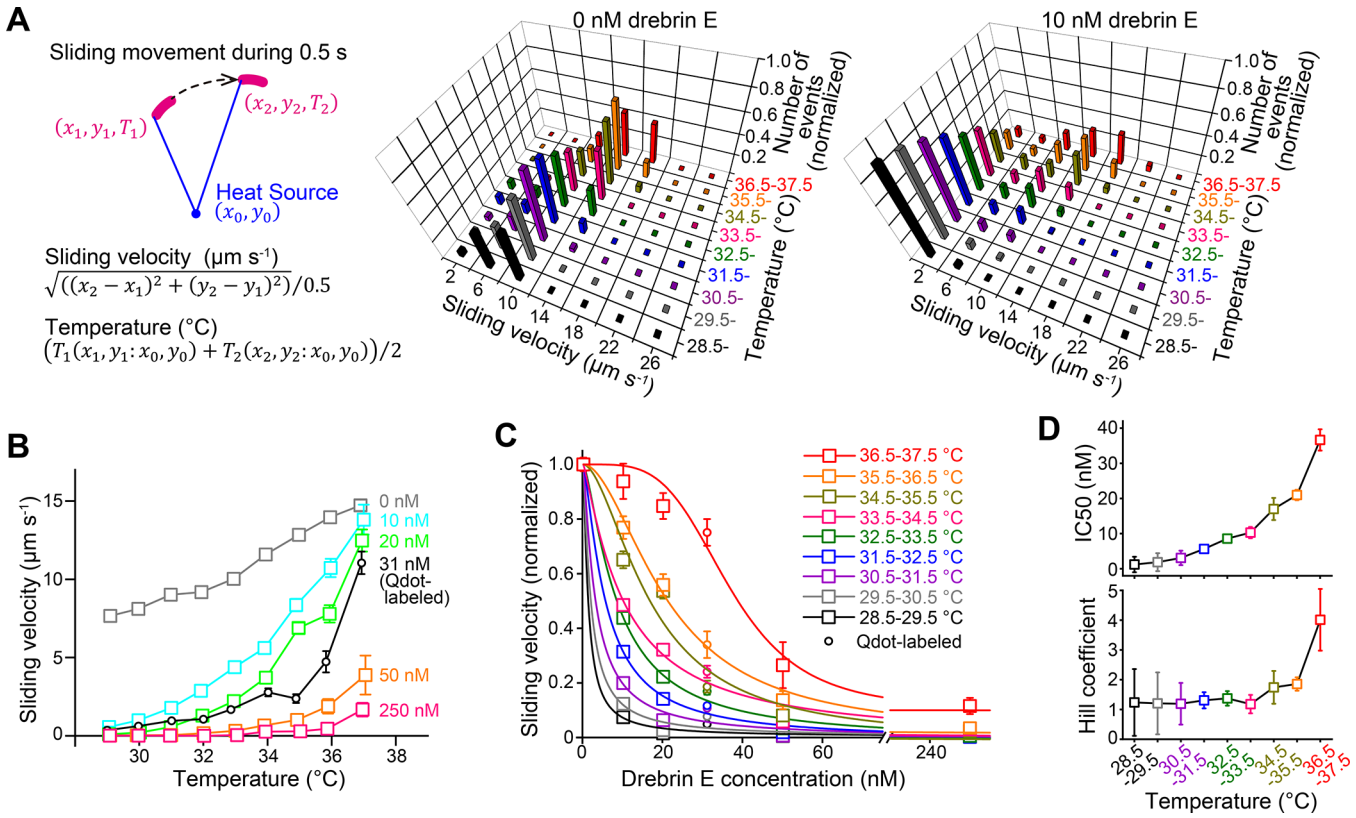

Figure 4. Temperature- and drebrin E concentration-dependent regulation of actomyosin motility (A, left) Schematic illustration of the procedure of sliding velocity and temperature determination of an actin filament. The distance covered by an actin filament during 0.5 s (5 frames) from one point (x_1, y_1) to another (x_2, y_2) was divided by time to determine the sliding velocity. Temperatures T_1 and T_2 at (x_1, y_1) and (x_2, y_2), respectively, were calculated from the temperature map (Figure 1) and the distance from the heat source located at (x_0, y_0), and then the average value of temperatures at (x_1, y_1) and (x_2, y_2) was defined as the temperature in this time range. The sliding velocity of immobile filaments is zero. (A, middle and right) Sliding velocity in the absence (middle, 0 nM) and in the presence of 10 nM (right) drebrin E in various temperature ranges. See Figure S6 regarding the sliding velocities at 0, 10, 20, 50, and 250 nM drebrin E and 31 nM Qdot-labeled drebrin E. The numbers of analyzed sliding events were 14 948, 17 306, 14 256, 6902, and 5845 for 0, 10, 20, 50, and 250 nM drebrin E, respectively, and 11 368 for 31 nM Qdot-labeled drebrin E. (B) Temperature dependence of sliding velocity at various drebrin E concentrations. Plots and error bars show means and standard error of the mean, respectively, in various temperature ranges. Plots were connected by lines to guide an eye. (C) Dependence of sliding velocity on drebrin E concentration in various temperature ranges. Plots and error bars show means and standard error of the mean, respectively. Curves are fittings with Hill equation $v = 1 - \frac{C^n(1-b)}{k^n + C^n}$, where v, C, k, n, and b represent the sliding velocity, drebrin E concentration, IC50, Hill coefficient, and baseline, respectively. See Table S1 for the determined values. (D) IC50 (top) and Hill coefficient (bottom) derived from the fits in (C) in various temperature ranges. Plots were connected by lines to guide an eye.

temperature range (Figure 4C). The change of the sliding velocity curve shape from decay-type to sigmoidal-type with the temperature increase is determined as (i) the nonlinear decrease in sliding velocity with drebrin E concentration and (ii) the higher concentration of drebrin E required at a higher temperature to gain an inhibitory effect. The data obtained in the presence of 31 nM Qdot-labeled drebrin E as in Figure 3 were combined with those in Figure 4B,C (open small circles). Although the results of the Qdot-labeled drebrin E at a lower temperature appear to be a little deviated from other data without labeling, the trend seems consistent within the accuracy of the current measurement. Therefore, we conclude that the Qdot-labeled drebrin E is effective in the motility assay.

The change of trend with temperature (Figure 4C) was described quantitatively by the half-maximum inhibitory concentration of drebrin (IC50) and Hill coefficients shown in Figure 4D and Table S1. The IC50 value that corresponded to half of the maximum velocity gradually increased from a few nanomolar up to over 30 nM of drebrin concentration as the temperature rose. The Hill coefficient, which reflects the degree of cooperativity in the given reaction, was kept slightly above 1.0 at a temperature below ~34 °C, whereas it abruptly increased to 4.0 at 36.5−37.5 °C.

DISCUSSION

The current study investigated the drebrin E-mediated modulation of actomyosin activity and its thermal sensitivity using the optically controlled microscopic heat pulse method. The observed reduction of drebrin-E mediated inhibition during heating would be simply attributed to the increase in the dissociation constant (Figure S4). According to our results, it was not the case, as the quick switching on/off of the regulation was observed in response to the heat pulses without the significant changes in the number of attached drebrin E molecules during heating (Figure 3). However, the rate of recording (0.1 s) could not detect the kinetics faster than 0.1 s. Therefore, our observation does not fully rule out the possibility of a rapid dissociation of drebrin E molecules from actin filaments and rebinding to them. Studies in muscle

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physiology have demonstrated that the affinity of actin and myosin[19,20] and the number of force-generating myosin molecules[21] are elevated by temperature rises. According to these reports, it is possible, at least partially, to explain the reduction of the drebrin E-mediated inhibition of actomyosin motility by a heat-enhanced interaction of actin filament and myosin molecules if, for example, especially the binding sites of drebrin E and myosin overlap on the actin filament.

Our data also demonstrated that the filament sliding was cooperatively inhibited while the drebrin E concentration increased at the physiological temperature (Figure 4C,D). The relatively higher degree of reaction cooperativity was recognized by the fitting even without the data on 31 nM Qdot-labeled drebrin E (Hill coefficient = 3.3). A previous study has reported the elongation of a helical half pitch of actin filament from 36 to 40 nm in the presence of 2 μM of the full length of drebrin A by its binding.[22] Moreover, the pitch increase propagates over up to two neighboring helical repeats on the actin filament in the presence of the actin-binding domain of drebrin A ($DrebrinA_{1-300}$) at the 3:1 molar ratio (actin/$DrebrinA_{1-300}$).[23] It has been also reported that the binding of $DrebrinA_{1-300}$ to actin filaments is cooperative at the room temperature and that the partial decoration was observed as clustering of $DrebrinA_{1-300}$ along the filaments (0.9 μM) in the presence of 280 nM $DrebrinA_{1-300}$.[23] The occupancy of the drebrin binding site on actin filament $p_{drebrin}$ is calculated as $p_{drebrin} = [drebrin]/(K_d + [drebrin])$, where [drebrin] is the concentration of free drebrin, and K_d is the dissociation constant of drebrin and actin filament. At $[DrebrinA_{1-300}] = 280$ nM and $K_d = 170$ nM drebrin A_{1-300},[24] $p_{DrebrinA_{1-300}} = 62\%$; hence, the partial decoration of an actin filament could induce the elongation of the filament. In the current study, we found that K_d is 6 and 32 nM at 25 and 37 °C, respectively (Figure S4). The value and its temperature-dependency are consistent with the IC50 value and its increase by the temperature rise (Figure 4D, top). The drebrin E-mediated inhibition of sliding was sufficiently effective when $p_{drebrin\ E} = 63\%$ by [drebrin E] = 10 nM at 25 °C (no sliding was observed as shown in Figure 2). However, with the similar occupancy (61%) by [drebrin E] = 50 nM, or even higher (89%) by [drebrin E] = 250 nM, the inhibition was reduced, and the sliding was observed at 37 °C (Figure 4B). Furthermore, the inhibition was almost fully reduced at 20 and 31 nM drebrin E at 37 °C (Figure 4B), where a substantial amount of the binding site remains occupied (38% and 49%, respectively). These experimental results support our suggestion that the reduced inhibition of actomyosin activity at higher temperatures could not be caused only by the dissociation of drebrin E from the actin filament, but drebrin E bound to the actin filament also regulates actomyosin activity more effectively at higher temperatures in a cooperative manner.

The structural modification (e.g., helical pitch changes) would affect the myosin binding sites not only sterically but also allosterically in the thermosensitive manner, hence, demonstrating the strong cooperativity in actomyosin regulation solely by the shift of drebrin E concentration at the physiological temperature. The regulatory protein of muscle contraction, tropomyosin-troponin complex, is a side-binding protein of actin filaments. It has been demonstrated that heating unfolds the coiled-coil domains of tropomyosin and decreases its affinity to actin.[25−27] As the other side-binding protein of actin filaments, drebrin E may have a similar mechanism for the temperature-dependent actomyosin regulation; that is, heating may reduce the affinity of some binding sites of drebrin E to the actin filament, partial dissociation of the molecule is caused while attaching to the actin filament, the inhibitory function is reduced, and actomyosin interaction is allowed. Direct evidence of this scenario can be provided if the residue(s) that regulates the actomyosin activity in a temperature-dependent manner is identified in the actin-binding sites of drebrin E,[24] and the structural shift of actin filaments decorated by drebrin E is visually observed by temperature changes. These are still future challenges.

The cooperative regulation by drebrin E concentration is suitable for the regulation of an actomyosin force generation in vivo; the force generation remains unaffected while the gene expression of drebrin E is inactive, whereas the inhibition starts immediately when the amount of protein exceeds a certain threshold. The concentration of drebrin E of ∼30 nM in the physiological condition can be estimated from the amounts of proteins purified from the brain of rat embryo (0.05 mg/20 g brain) while the molecular weight of drebrin E is assumed to be 77 kDa.[5] This value matches well with the concentration when actomyosin inhibition was strong at 36.5−37.5 °C, as shown in this work (Figure 4B,C).

In conclusion, our study revealed the cooperative behavior in the actomyosin regulation by drebrin E, which is remarkable only at the physiological temperature. Our results suggest the importance of maintaining the body temperature of mammal mothers during pregnancy for the precise development of an embryo. Harris et al. recently demonstrated that the changes of the actin filament conformation by $DrebrinA_{1-300}$ alter the binding kinetics of utrophin actin-binding domain mutants.[28] In addition to the competitive binding as a simple mechanism for the regulation of actomyosin activity, a modulation of the structure of an actin filament in a cooperative manner may be a common ability for other side-binding proteins of the actin filament. The ability of these allosteric inhibitors should support the actomyosin mechanical forces to drive a wide variety of cellular functions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.1c02955.

Materials and methods, the values of fitting parameters by the Hill equation, temperature dependence of the fluorescence intensity of rhodamine−phalloidin measured by fluorescence spectrometer, correction of the baseline shift due to photobleaching, ratio of sliding actin filaments at each time point after a heat pulse, cosedimentation assay of drebrin E with actin filaments, effects of preincubation on the ratio of mobile actin filament, sliding velocities of actin filaments at various drebrin E concentrations (PDF)

Movie S1. Temperature map during heating by 1455-nm infrared laser light (MP4)

Movie S2. Fluorescence imaging of actin filaments in the in vitro motility assay without drebrin E (MP4)

Movie S3. Fluorescence imaging of actin filaments in the in vitro motility assay in the presence of 10 nM drebrin E (MP4)

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Movie S4. Fluorescence imaging of actin filaments in the in vitro motility assay in the presence of 20 nM drebrin E (MP4)

Movie S5. Fluorescence imaging of actin filaments in the in vitro motility assay in the presence of 50 nM drebrin E (MP4)

Movie S6. Fluorescence imaging of actin filaments in the in vitro motility assay in the presence of 250 nM drebrin E (MP4)

Movie S7. Fluorescence imaging of actin filaments with Qdot-labeled drebrin E in the in vitro motility assay (MP4)

Movie S8. Fluorescence imaging of Qdot-labeled drebrin E on actin filaments in the in vitro motility assay (MP4)

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Author Contributions

H.K., H.O., M.M., S. Ishiwata, and M.S. designed the experiments. H.K., H.O., M.M., S. Ishii, K.O., and Y.K. performed experiments and analyzed data. K.O. and M.S. set up the microscope. H.K., H.O., M.M., S. Ishiwata, and M.S. wrote the manuscript, which was approved by all the authors.

Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

F.I.: Fluorescence intensity
HMM: Heavy meromyosin

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