Visualizing myosin–actin interaction with a genetically-encoded fluorescent strain sensor

Sosuke Iwai,a,1 and Taro Q. P. Uyeda,a,b,1

aResearch Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan; and bBiomedical Information Research Center, National Institute of Advance dsIndustrial Science and Technology, 2-42 Aomi, Koto, Tokyo 135-0064, Japan

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Many proteins have been shown to undergo conformational changes in response to externally applied force in vitro, but whether the force-induced protein conformational changes occur in vivo remains unclear. To reveal the force-induced conformational changes, or strains, within proteins in living cells, we have developed a genetically encoded fluorescent “strain sensor,” by combining the proximity imaging (PRIM) technique, which uses spectral changes of 2 GFP molecules that are in direct contact, and myosin–actin as a model system. The developed PRIM-based strain sensor module (PriSSM) consists of the tandem fusion of a normal and circularly permuted GFP. To apply strain to PriSSM, it was inserted between 2 motor domains of Dictyostelium myosin II. In the absence of strain, the 2 GFP moieties in PriSSM are in contact, whereas when the motor domains are bound to F-actin, PriSSM has a strained conformation, leading to the loss of contact and a concomitant spectral change. Using the sensor system, we found that the position of the lever arm in the rigor state was affected by mutations within the motor domain. Moreover, the sensor was used to visualize the interaction between myosin II and F-actin in Dictyostelium cells. In normal cells, myosin was largely detached from F-actin, whereas ATP depletion or hyperosmotic stress increased the fraction of myosin bound to F-actin. The PRIM-based strain sensor may provide a general approach for studying force-induced protein conformational changes in cells.

Optimization of GFP Concatemer for PRIM. We first sought to optimize GFP concatemer for the PRIM technique. In the first report on PRIM (15), 2 natural GFP molecules were joined in tandem to provide a test case for PRIM, but the spectral changes caused by the covalent linking were relatively small. The crystal

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Many proteins have been shown to undergo conformational changes in response to externally applied force in vitro (1). They include structural proteins, such as muscle and cytoskeletal proteins, which are responsible for maintaining the structural integrity of cells (2–5). On the other hand, mechanosensitive proteins, which include cell adhesion proteins and proteins linked to mechanosensitive ion channels, are involved in the transduction of mechanical signals to cells (6–8). In either case, protein conformational changes are believed to play important roles in the processes, although whether they occur in vivo remains unclear. Recently, evidence has accumulated for force-induced protein conformational changes in cells. For instance, the Src family kinase substrate is mechanically extended in spread cells, as revealed by conformation-sensitive antibody (9). More recently, a shotgun cysteine labeling approach revealed that several cytoskeletal proteins change their conformation or assembly in mechanically stressed cells (10). Despite these studies, little is known about the spatial and temporal dynamics of the protein conformational changes in cells. To reveal the force-induced conformational changes, or strains, within proteins in living cells, a polypeptide-based fluorescent “strain sensor” would be necessary.

Fluorescent resonance energy transfer (FRET) is a technique that can measure the proximity or distance between a donor and an acceptor molecule. FRET has been widely used to detect protein conformational changes, in particular, when combined with various fluorescent proteins (11). However, FRET has a limitation in resolving protein conformational changes in cells. FRET efficiency is usually estimated from the fluorescent intensity of samples (12), but it is known that proteins often exist in multiple conformational states (13), and the intensity-based FRET method cannot distinguish between a conformational intermediate state and a mixture of multiple states. Fluorescence lifetime microscopy combined with multicomponent analysis (14) or single-molecule imaging (13) may overcome this problem, but demand complicated apparatus and analysis. A decade ago, De Angelis et al. (15) reported another GFP-based technique, which they termed proximity imaging (PRIM). PRIM depends on direct contact between 2 GFP molecules, which can lead to structural perturbations and concomitant spectral changes (15, 16). Unlike FRET, PRIM is assumed to involve only 2 types of fluorescent excitation spectra corresponding to monomeric and dimeric GFP, so that an estimated excitation ratio will simply reflect a mixing ratio of the monomer and the dimer, in principle. Therefore, PRIM would be useful for detecting the protein conformational changes in vitro and in vivo.

Myosin is an actin-based motor protein that undergoes cyclic interaction with F-actin during the ATP-hydrolysis cycle. In a widely accepted model, the driving force for motion is generated as a result of conformational changes in the motor domain while attaching to F-actin (17). Such conformational changes cause distortion of an elastic element within the myosin molecule and allow strain to develop (18), which can lead to relative displacement of myosin and F-actin. Thus, the myosin and actin system is a molecular strain generator and can be used to apply strain to the polypeptide-based sensor in solution. In this study, we have used Dictyostelium myosin II and actin as a model system to characterize the strain sensor. By combining the GFP-based PRIM technique and myosin–actin as the model system, we developed a genetically encoded fluorescent sensor that can detect force-induced conformational changes, or strains, within proteins. Using the sensor system, we found that the position of the lever arm in the rigor state was affected by mutations within the motor domain. Moreover, the sensor was used to visualize the interaction between myosin II and F-actin in Dictyostelium cells.

Results

Optimization of GFP Concatemer for PRIM. We first sought to optimize GFP concatemer for the PRIM technique. In the first report on PRIM (15), 2 natural GFP molecules were joined in tandem to provide a test case for PRIM, but the spectral changes caused by the covalent linking were relatively small. The crystal

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1To whom correspondence may be addressed. E-mail: iwai-sosuke@aist.go.jp or t-uyeda@aist.go.jp.

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A 490/390-nm fluorescence excitation ratio, R values. GFP or cp174GFP alone are monomeric GFP derivatives. Between 2 motor domains of Myosin II (PriSSM) were joined with a minimized linker. In the absence of external force, the 2 GFP moieties are expected to be in contact and show a fluorescence excitation spectrum similar to that of the GFP dimer. In the presence of both F-actin and ATP, the 2 GFP moieties are expected to lose the contact between the GFP moieties and show a fluorescence excitation spectrum similar to that of the GFP monomers or monomeric derivatives.

Characterization of a Strain Sensor Using Myosin-Actin as a Model System. To apply strain to PriSSM, the module was inserted between 2 motor domains of Dictyostelium myosin II (PriSSM-motor). As a result, PriSSM-motor consists of a tandem fusion of an N-terminal motor domain, the sensor module PriSSM containing the 2 GFP moieties, and a C-terminal motor domain (Fig. 24). The C terminus of the myosin motor domain is a long α-helix and, with bound 2 light chains, thought to act as a lever arm that amplifies conformational changes that occur in the catalytic domain (17, 23, 24). To efficiently transmit these conformational changes to the sensor module, the α-helix at the C terminus of the N-terminal motor domain was directly joined to the C terminus of the other protomer in the crystal structure of dimer, because linking the nearest ends would have minimum steric effect on the dimer formation. cp174GFP, which contains a new N terminus at Gly-174, was joined with normal GFP with a 29-aa flexible linker to produce GFP-29-cp174GFP (Fig. 1A). The fluorescence excitation spectrum of GFP-29-cp174GFP was measured and compared with that of an equal mixture of GFP and cp174GFP (Fig. 1B). Notably, GFP-29-cp174GFP showed a peak at 390 nm similar to the one observed for an absorption spectrum of dimeric GFP (22). Fig. 1C shows a 490/390-nm fluorescence excitation ratio, R 490/390, for GFP-29-cp174GFP and other GFP concatemers or monomeric derivatives. The GFP-29-cp174GFP showed a strikingly lower R 490/390 than that of the monomers or of the concatemers consisting of 2 normal GFP molecules. The length of the linker between the 2 GFP moieties had only slight effects on R 490/390. Because GFP-29-cp174GFP showed 1 of the largest spectral changes as compared with monomeric GFP, GFP-29-cp174GFP was tested as a PRIM-based strain sensor module (PriSSM).
a fluorescence property similar to that of monomeric GFP. Upon addition of ATP, the motor domains would detach from F-actin, which can lead to the relief of the strain and a concomitant reversal of the spectral change.

PriSSM-motor was expressed in Dictyostelium cells and purified by ATP extraction of a Triton-insoluble cytoskeleton fraction and nickel affinity chromatography using the His tag fused to the C terminus of the protein. The purified protein changed the fluorescence excitation spectra in actin and/or ATP-dependent manners (Fig. 2C). As expected, \[ R_{490/390} \] increased >2-fold upon addition of F-actin (Fig. 2D, PriSSM-motor). The \[ R_{490/390} \] in the absence and presence of actin were comparable to that of the GFP concatemer and monomer, respectively. This finding suggested that, when both of the motor domains formed rigor complexes with F-actin, the protein was in a strained conformation and the 2 GFP moieties lost their contact. This process was ATP-independent, suggesting that the intramolecular association between the 2 GFP moieties was disrupted by thermal activation without the help of active force developed by the myosin motor. \[ R_{490/390} \] decreased when ATP was added and then increased again when apyrase was added, suggesting that the conformational and spectral changes of PriSSM-motor were reversible. In the presence of both ADP and actin, PriSSM-motor showed an \[ R_{490/390} \] similar to that in the rigor state, consistent with the model that the myosin motor domain attached to F-actin does not undergo a major conformational change accompanying ADP release (17). Collectively, the PriSSM-motor showed high and low values for \[ R_{490/390} \] (dynamic range, >100%), corresponding to the strained and unstrained states, respectively, confirming that PriSSM functions as a strain sensor module.

When PriSSM-motor is bound to F-actin in the absence of ATP, the lever arm of the N-terminal motor domain would be in the poststroke position with regard to F-actin, which may produce the strained state of the sensor. To confirm this idea, 3 residues at the base of the lever arm within the N-terminal motor domain (Ile-766, Lys-767, Ala-768) were replaced with glycine residues (Fig. 2A, PriSSM-GGG). The change in \[ R_{490/390} \] for PriSSM-GGG on addition of F-actin was significantly smaller than that for PriSSM-motor (Fig. 2D), supporting the idea that the lever arm is in the poststroke orientation to F-actin when the sensor is in the strained state, and also suggesting that the rigidity of the lever arm is important for its orientation. Strong binding of the motor domain to F-actin is also thought to be important for the lever arm conformation (17). To examine whether the lever arm position in PriSSM-motor depends on the strong binding, the cardiomypathy loop within the N-terminal motor domain, which is involved in the strong binding (26), was deleted (Fig. 2A, PriSSM-ΔCMN). The deletion caused a smaller change in \[ R_{490/390} \] (Fig. 2D), suggesting that the strong binding to F-actin is important for maintaining the position of the lever arm relative to F-actin. Likewise, the deletion of the loop within the C-terminal motor domain (Fig. 2A, PriSSM-ΔCMC) also caused a smaller change in \[ R_{490/390} \] (Fig. 2D). The results of the cardiomypathy loop deletions together suggest that, when the sensor is in the strained state with a high \[ R_{490/390} \] both of the motor domains are mostly in the strongly bound state.

**Visualization of Myosin–Actin Interaction in Cells.** PriSSM-motor showed the ratiometric and reversible spectral changes when attached to F-actin, allowing us to detect the interaction of myosin with F-actin in cells by using the sensor. To detect the behavior of intact myosin II, the C-terminal His tag of PriSSM-motor was eliminated and replaced with the myosin tail domain to produce PriSSM-myosin (Fig. 3A). To examine whether PriSSM-myosin retains physiological functions, the protein was expressed in myosin II-null cells. Dictyostelium myosin II-null cells are unable to divide in suspension culture or form fruiting bodies when starved, providing evidence that myosin II is involved in cytokinesis and morphogenesis (27, 28). Myosin II-null cells expressing PriSSM-myosin divided normally in suspension culture (data not shown) and developed completely to form normal fruiting bodies comparable to those formed by the cells expressing wild-type myosin II (Fig. 3B). These results suggest that PriSSM-myosin retains physiological functions for myosin II and is expected to behave like native myosin II in cells. For imaging studies, PriSSM-myosin was expressed in wild-type Dictyostelium cells to form a heterodimer with endogenous myosin II, to reduce nonspecific GFP-GFP interactions, which might occur in homodimeric PriSSM-myosin.

The cells expressing PriSSM-myosin were suspended in buffer and measured for fluorescence excitation spectra (Fig. 4A Upper Left). Because the measured spectrum contained a considerable amount of autofluorescence of cells, the autofluorescence was measured independently by using control Dictyostelium cells, and the contributions from PriSSM-myosin and the autofluorescence were separated by means of a linear unmixing procedure (29). The contribution from PriSSM-myosin was composed mostly of the spectrum of the purified PriSSM-motor in the presence of ATP with the low \[ R_{490/390} \] value. This finding suggests that at least 1 of the 2 motor domains of PriSSM-myosin is mostly detached from F-actin in cells, because the sensor would show a high \[ R_{490/390} \] value if both of the motor domains are bound. When Dictyostelium cells are treated with sodium azide, which depletes cellular ATP, they round up and contract (30). Fluorescence excitation spectrum of PriSSM-myosin in azide-treated cells was also measured (Fig. 4A Upper Right). In contrast to the cells in normal buffer, azide-treated cells showed relatively high \[ R_{490/390} \] values (Fig. 4A Lower Right), suggesting that the ATP depletion increased the fraction of PriSSM-myosin that was bound to F-actin. Similarly, addition of 350 mM sorbitol significantly increased \[ R_{490/390} \] (Fig. 4A Lower Right), suggesting that hypertonic stress also increased the fraction of PriSSM-myosin bound to F-actin. Although these external stimuli reduced the fluorescence intensity of the sensor probably by acidifying the cytosol (31), \[ R_{490/390} \] of PriSSM was not affected by pH changes between pH 6 and 8 (supporting information [SI] Fig. S1).

Triton-insoluble cytoskeletons, or Triton ghosts, contain actin and myosin II as major components (32, 33) and are suitable for initial microscopic observations of PriSSM-myosin. The cells expressing PriSSM-myosin were attached onto a glass surface and lysed with 0.5% Triton X-100 to obtain Triton ghosts containing PriSSM-myosin. Fluorescent images of the Triton ghosts were acquired under excitation at 380 ± 15 or 480 ± 15...
nm to estimate 480/380-nm excitation ratios, \( R_{480/380} \). The spectroscopic experiments of PriSSM showed that \( R_{480/380} \) was similar to \( R_{490/390} \). The \( R_{480/380} \) of the ghosts were high in the absence of nucleotides (Fig. 4B Left). Upon addition of ATP, the ghosts contracted rapidly and \( R_{480/380} \) decreased significantly (Fig. 4B Center), suggesting that some PriSSM-myosin was detached from F-actin. PriSSM-myosin\( \Delta N \) lacks the N-terminal motor domain (Fig. 3A) and would not be strained even if attached to F-actin. As expected, PriSSM-myosin\( \Delta N \) showed a low \( R_{480/380} \) value in the absence of nucleotides (Fig. 4B Right). These results suggest that PriSSM-myosin showed fluorescent changes sufficient for microscopic observations reflecting interaction with F-actin.

Then we observed PriSSM-myosin in living cells. To minimize the background of autofluorescence, the cells were starved in buffer for 3 h and then compressed by thin agarose sheets (34). In this condition, myosin filaments are specifically enriched in cortical regions (34, 35) and can be microscopically separated from autofluorescence, which localize mainly to vesicular structures. However, because of residual autofluorescence, fluorescence ratio values obtained in living cells remained qualitative in our current experimental system. In the condition described above, the \( R_{480/380} \) of PriSSM-myosin was relatively low (Fig. 4C Left), further supporting the finding that at least 1 of the 2 motor domains of PriSSM-myosin was mostly detached from F-actin in normal cells. The \( R_{480/380} \) of PriSSM-myosin remained low even in cells undergoing cytokinesis or chemotaxis (Fig. S2). To deplete cellular ATP for imaging studies, cells were treated with 2,4-dinitrophenol (DNP) instead of azide, because azide tended to reduce the fluorescence intensity of the sensor as described above. In DNP-treated cells, most F-actin is separated from the plasma membrane and accumulated in a layer beneath the cell surface (36). In those cells, PriSSM-myosin showed high \( R_{480/380} \) values in the areas beneath the cell surface, where F-actin was shown to accumulate (Fig. 4C Center), suggesting that the ATP depletion caused PriSSM-myosin to be bound to F-actin and accumulate. The \( R_{480/380} \) of PriSSM-myosin\( \Delta N \) remained low even in DNP-treated cells (Fig. 4C Right), indicating that high \( R_{480/380} \) values for PriSSM-myosin obtained in DNP-treated cells were not ascribed to unusual accumulation of the fluorescent proteins.

**Discussion**

We have developed a polypeptide-based fluorescent strain sensor, using the GFP-based PRIM technique. The optimized strain sensor module (PriSSM) consists of a tandem fusion of a normal GFP and a cpGFP and shows the 2 types of fluorescence excitation spectra corresponding to the strained and unstrained states. Although the detailed mechanism of the spectral changes still remains to be determined, PRIM is assumed to involve only 2 types of spectra for a given GFP concatemer, so that an estimated excitation ratio would in principle reflect a mixing ratio between the strained and unstrained states. The sensor shows not only rational but also reversible fluorescence changes, and thereby can be visualized in living cells continuously by using dual excitation fluorescence microscopy. As a model system to characterize the sensor, we used Dictyostelium myosin II and actin, a molecular strain generator. The system can apply strain to the sensor module in solution and thereby provides a convenient assay for developing fluorescent strain sensors. Also, the sensor may provide a simple assay for examining the effects of mutations within the myosin motor domain on the lever arm position. The mutational studies suggest that both the lever arm rigidity and the strong binding to F-actin are important for maintaining the poststroke position of the lever arm relative to F-actin, which produces the strained state of the sensor. These results are consistent with the model that the optimal force generation requires the rigidity of the lever arm and the strong binding to F-actin (17, 26, 37).

The developed sensor was used to visualize the interaction between myosin and F-actin in Dictyostelium cells. Both spectroscopic and microscopic studies suggest that the fraction of PriSSM-myosin that is bound to F-actin is low in normal cells. In vitro studies of PriSSM-motor suggested that, when the sensor was in the strained state, the 2 motor domains were mostly in the strongly bound state. Therefore, the probability of the strained state would be approximately a square of the binding probability of the single motor domain, assuming that the 2 motor domains bind to F-actin independently. From the spectroscopic experiments, the probability of PriSSM-myosin in the strained state was <1% in normal cells, so that the binding probability of the
single motor domain would be at most <10%. This idea is consistent with the notion that the nonmuscle myosin II proteins are largely detached from F-actin in cells, which is explained by the 2 previously reported properties of the protein. First, most of the Dictyostelium myosin II proteins normally exist as a monomer in the cytoplasmic pool (38), which is separated from F-actin-enriched cellular structures. Second, under the cellular concentration of ATP, myosin II is a typical motor with a low duty ratio and is mostly detached from F-actin, even when assembled into thick filaments (39). In contrast to the normal cells, cells treated with azide or DNP, which deplete cellular ATP, showed an increase in the fraction of the motor domain that was bound to F-actin. This outcome suggests that the contraction of cells by ATP depletion results from the increased binding of myosin II. Under hypotonic conditions, Dictyostelium cells are known to rearrange their cytoskeletal proteins including myosin II and actin (40, 41). We found that hypotonic stress also increased the fraction of the motor domain bound to F-actin, which may be at least partly caused by the decrease in the cellular nucleoside triphosphate level under hypertonic conditions (31). The increased binding of myosin II may contribute to maintaining cortical tension to protect cells against the osmotic stress.

When the 2 motor domains of PriSSM-motor were bound to F-actin, the intramolecular association between the 2 GFP moieties was disrupted by thermal activation without the help of active force developed by the myosin motor. The separated state was maintained stably in the presence of F-actin and in the absence of ATP, suggesting that the bond strength between the GFP moieties is smaller than that between myosin and F-actin, of which unbinding force is several piconewtons. Thus, our PRIM-based sensor, which is at least partly caused by the decrease in the cellular nucleoside triphosphate level under hypertonic conditions (31). The increased binding of myosin II may contribute to maintaining cortical tension to protect cells against the osmotic stress.

Materials and Methods

Expression and Purification of Proteins. For expression of GFP concatemers, cDNAs encoding GFP or the cpGFP both carrying the F64L/S65T mutations (46) were amplified by PCR and ligated into pET30b(+) (Novagen) with linker sequences containing GGGS repeats. cDNA encoding cpGFP with the N terminus at Gly-174 was amplified according to ref. 48. All GFP-derived proteins were expressed in Escherichia. coli Rosetta(DE3) (Novagen) at 22 °C for 7 h. The proteins were purified by using a Ni-nitrotriacetic agarose (Qiagen) column and dialysed against an assay buffer containing 100 mM Hepes (pH 7.4), 50 mM KCl, MgCl₂, and 1 mM DTT. In some cases, the proteins were further incubated overnight at room temperature to facilitate protein folding and chromatophore formation. Concentrations of the GFP-derived proteins were determined by the Bradford method using GFP as a standard.

For expression of PriSSM-motor and PriSSM-myosin, cDNAs encoding a Dictyostelium myosin II heavy chain fragment (residues 1–768), the GFP concatemer with a 29-aa linker, and the other myosin heavy chain fragment (residues 3–761 or 3–2116) were ligated into pET30b(+) (Novagen) with linker sequences containing GGGS repeats. These modifications may increase the unbinding forces among 2 previously reported properties of the protein. First, most of the Dictyostelium myosin II proteins normally exist as a monomer in the cytoplasmic pool (38), which is separated from F-actin-enriched cellular structures. Second, under the cellular concentration of ATP, myosin II is a typical motor with a low duty ratio and is mostly detached from F-actin, even when assembled into thick filaments (39). In contrast to the normal cells, cells treated with azide or DNP, which deplete cellular ATP, showed an increase in the fraction of the motor domain that was bound to F-actin. This outcome suggests that the contraction of cells by ATP depletion results from the increased binding of myosin II. Under hypotonic conditions, Dictyostelium cells are known to rearrange their cytoskeletal proteins including myosin II and actin (40, 41). We found that hypotonic stress also increased the fraction of the motor domain bound to F-actin, which may be at least partly caused by the decrease in the cellular nucleoside triphosphate level under hypertonic conditions (31). The increased binding of myosin II may contribute to maintaining cortical tension to protect cells against the osmotic stress.

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sheet (34) and incubated for another 30 min. To make observation chambers, 2 strips of 0.2-mm-thick filter paper were placed along the sides of the agarose sheet as spacers. The agarose and the filter paper were enclosed by a layer of silicon grease and then covered with a second glass coverslip (22 × 22 mm).

The Triton ghosts or the cells were observed at 22° C by using an Olympus IX71 inverted microscope equipped with a 100× UPlanApo oil-immersion objective. For excitation, light from the mercury lamp was reduced to 25% and selected with a D380/30× or D480/30× band-pass filter (Chroma). Fluorescence images were obtained through a 500DCXR dichromatic mirror and a D535/40m band-pass filter (Chroma) and collected on a cooled charge-coupled device camera ORCA-ER (Hamamatsu) controlled by IPLab software (Scanco). The images were analyzed by using a customized plugin for ImageJ (http://rsb.info.nih.gov/ij/).

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Fig. S1. pH dependence of fluorescence properties of PriSSM (GFP-29-cp174GFP). Spectra of the purified protein were measured at a protein concentration of 0.1 μM in a buffer containing 50 mM NaH₂PO₄ and 50 mM KCl, adjusted to the indicated pH values with NaOH. (A) Fluorescent excitation spectra of PriSSM between pH 6 and 8. (B) pH dependence of $R_{490/390}$ of PriSSM. The pH changes affected the fluorescence intensity but not the $R_{490/390}$ of PriSSM between pH 6 and 8.
Fig. S2. Observations of cells expressing PriSSM-myosin. The cells were observed as described in Materials and Methods. R\textsubscript{480/380} was color-encoded according to the look-up table. (A) A dividing cell. (B) Chemotaxing cells. To observe chemotaxing cells, the cells were incubated in 10 mM Mes (pH 6.8) for 8 h before the observations. The R\textsubscript{480/380} remained low even in cells undergoing these events. (Bars: 10 \(\mu\)m.)