D-loop of Actin Differently Regulates the Motor Function of Myosins II and V**§

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To gain more information on the manner of actin-myosin interaction, we examined how the motile properties of myosins II and V are affected by the modifications of the DNase I binding loop (D-loop) of actin, performed in two different ways, namely, the proteolytic digestion with subtilisin and the M47A point mutation. In an in vitro motility assay, both modifications significantly decreased the gliding velocity on myosin II-heavy meromyosin due to a weaker generated force but increased it on myosin V. On the other hand, single molecules of myosin V “walked” with the same velocity on both the wild-type and modified actins; however, the run lengths decreased sharply, correlating with a lower affinity of myosin for actin due to the D-loop modifications. The difference between the single-molecule and the ensemble measurements with myosin V indicates that in an in vitro motility assay the non-coordinated multiple myosin V molecules impede internal friction on each other via binding to the same actin filament, which is reduced by the weaker binding to the modified actins. These results show that the D-loop strongly modulates the force generation by myosin II and the processivity of myosin V, presumably affecting actin-myosin interaction in the actomyosin-ADP-Pi state of both myosins.

Subdomain 2 of actin, which contains the D-loop (residues 38–52), slightly changes its conformation during actin polymerization and interacts with the C terminus of the adjacent subunit in actin filament (1, 2). This region is suggested to be important for actin-myosin interaction; it was found that binding of myosin II induces conformational changes in subdomain 2 (3, 4), whereas the proteolytic digestion of the D-loop inhibits actin-activated ATPase of myosin II-subfragment 1 (S1)3 and decreases the velocity of actin filaments on myosin II-HMM in an in vitro motility assay (5, 6).

However, although as many as 24 classes of myosin have already been found (7), the contribution of the D-loop to actin-myosin interaction has so far been studied only for myosin II. Although all myosins carry out their motor functions by interacting with actin filaments, each class has a different role in vivo. For example, myosin V is an intracellular transporter, which is different from the role of myosin II, engaged mainly in muscle contraction or the formation of contractile ring. The rate-limiting step in the ATPase cycle in the presence of actin is the phosphate release in the case of myosin II but ADP release in myosin V, which allows this motor to spend most of its ATPase cycle (>90%) strongly bound to actin. Furthermore, similarly to kinesin (8), two heads of myosin V work cooperatively, coordinating their biochemical cycles via internal load (9–11). These characteristics enable myosin V to move processively on actin filaments (12), and recent studies indicate that, similarly to myosin II, the attachment of myosin V also induces conformational changes in the D-loop region of actin (13).

Here, to determine whether the D-loop contributes to the interaction with myosin V and, if so, in what way it affects its motor function, we prepared actins modified in the D-loop and analyzed the effects of modifications on the motile properties of myosins II and V. The D-loop was modified by 1) subtilisin digestion of rabbit skeletal actin (sub-actin) and 2) site-directed point mutagenesis of Dictyostelium actin (Fig. 1A).

EXPERIMENTAL PROCEDURES

Plasmid Construction—The pBIG plasmid, which contained a neomycin-resistant gene and the complete Dictyostelium actin 15 sequence (14) with the E360H mutation, was a gift from Prof. T. Wakabayashi (Teikyo University). The M47A mutation was introduced to it by using the QuikChange site-directed mutagenesis kit (Qiagen) and the primer set: the forward primer (CGTCCAAGACACACTTGTTATGTTGTTGCGG) and the reverse primer (GACCCGCACCAACCACATACCCAGTAGTTGCTGGGTC) and the reverse primer (GACCCGCACCAACCACATACCCAGTAGTTGCTGGGTC) and the reverse primer (GACCCGCACCAACCACATACCCAGTAGTTGCTGGGTC). Whereas the M47A mutation was used for the functional analysis of the properties of the D-loop–modified actin, the E360H mutation, which does not significantly affect any important function of actin (14, 15), served as a purification tag, producing the difference in the surface charge compared with the endogenous wild-type actin; however, the difference in the surface charge due to the M47A mutation alone was insufficient. When M47A/E360H actin was functionally analyzed, the control measurements with E360H

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S5.

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§ The abbreviations used are: S1, subfragment 1; HMM, heavy meromyosin; sub-actin, subtilisin-cleaved actin; MMV, murine myosin V; MOPS, 4-morpholinepropanesulfonic acid.
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actin were performed during each experiment to confirm that no function of actin was compromised by the E360H mutation.

For the preparation of the recombinant myosin V, the sequence encoding first 913 (for MMV-S1) or 1091 (for MMV-HMM) residues of mouse myosin V heavy chain was ligated into pFastBac-HT vector (Invitrogen). The sequence encoding the light chain (human calmodulin) was ligated into pFastBac-1 vector (Invitrogen). DH10Bac-competent cells (Invitrogen) were transformed with these vectors to produce the recombinant bacmids, which were used for the transfection of Sf9 insect cells to obtain the baculoviruses encoding the heavy and the light chains of MMV-S1 and MMV-HMM.

Preparation of Actins—Recombinant Dictyostelium actins were prepared from the transfected cells as in Sutoh et al. (15) (see supplemental material for details). Sub-actin was prepared by digesting rabbit skeletal actin as follows. First, rabbit skeletal actin was purified from rabbit white skeletal muscle (16), instantly frozen in liquid nitrogen, and stored at −80 °C. All experimental procedures conformed to the Guidelines for Proper Conduct of Animal Experiments approved by the Science Council of Japan and were performed according to the Regulations for Animal Experimentation at Waseda University. Prior to subtilisin digestion, actin was thawed at room temperature and dialyzed overnight at 4 °C in G-buffer (2 mM Tris-HCl (pH 8.0), 0.05 mM CaCl₂, 2 mM NaCl, 0.5 mM mercaptoethanol, and 0.1 mM ATP). After dialysis, actin was digested by the addition of 1:1000 (w/w) subtilisin (Sigma) and incubation for 1 h at 25 °C (5). The reaction was stopped by the addition of 2 mM phenylmethylsulfonyl fluoride. The digestion was confirmed by SDS-PAGE. For an in vitro motility assay and a single-molecule myosin V movement assay, both wild-type and modified rabbit skeletal and Dictyostelium G-actins were polymerized by the addition of 100 mM KCl, 2 mM MgCl₂, 2 mM MOPS (pH 7.0), and 1.5 mM NaN₃. The resulting F-actins (2.34 μM) were stained with 6.6 μM rhodamine-phalloidin (Invitrogen) for an in vitro motility assay or with 3.3 μM rhodamine-phalloidin and 3.3 μM biotin-XX phalloidin (Invitrogen) for a single-molecule myosin V movement assay.

Preparation of Myosins—Myosin II was prepared from rabbit skeletal muscle and digested by chymotrypsin to obtain HMM (17). Native myosin V was purified from chick brain (18). Recombinant myosins (MMV-HMM and MMV-S1) were purified from the baculovirus-infected Sf9 cells by affinity and anion exchange chromatography (see supplemental material for details). To distinguish the full-length native myosin V and the recombinant myosin V, here the former is referred to as “myosin V” and the latter as “MMV-HMM” or “MMV-S1.”

In Vitro Motility Assay and the Sliding Force Measurement—An in vitro motility assay was performed on myosin II-HMM (19) or myosin V (12, 20) (see supplemental material for details). A filament that moved with its whole length at least once within 5 s, even if it was not moving continuously, was counted as “mobile.” All other filaments, including those of which only part was moving, even if continuously for 5 s, were counted as “immobile.” Additionally, the force generated by multiple myosin II-HMM molecules during the sliding movement of an actin filament was measured by optically trapping the gelsolin-coated bead attached to the trailing (barbed) end of an actin filament (21) (see supplemental material for details).

Single-molecule Myosin V Movement Assay—As in our previous study (22), native myosin V molecules were attached to the fluorescent polystyrene beads (diameter 0.2 μm, carboxylate-modified, yellow-green (505/515); Invitrogen). Myosin V molecules (molecular mass 650 kDa) and beads were mixed at a 0.75:1 molar ratio in A-buffer (25 mM imidazole-HCl (pH 7.4), 4 mM MgCl₂, 1 mM EGTA) containing 10 mg/ml bovine serum albumin and 300 mM KCl and incubated for 30 min on ice. By statistical analysis using optical tweezers, we previously confirmed that even a 3:1 molar ratio was sufficiently low to regard the movement of beads as that of single myosin V molecules (22). 5 mg/ml biotinylated bovine serum albumin in B-buffer (A-buffer containing 100 mM KCl) was infused into the flow cell (~16 μl), which was identical to that used in an in vitro motility assay, but without nitrocellulose coating, and incubated for 3 min. After washing with 40 μl of B-buffer, 40 μl of B-buffer containing 0.5 mg/ml streptavidin was infused and incubated for 3 min. After another wash with 40 μl of B-buffer, 40 μl of the solution of actin filaments (46.8 nm), stained with rhodamine-phalloidin and biotin-XX phalloidin, in B-buffer was infused and incubated for 10–18 min. Next, 40 μl of B-buffer containing 10 mg/ml bovine serum albumin and 127 nm non-labeled phalloidin was infused and incubated for 10–18 min. Finally, A-buffer containing 0.83 mM (for the experiments at 100 or 150 mM KCl) or 0.16 mM (at 30 mM KCl) beads, 1 mM ATP, 10 mM dithiothreitol, 10 mg/ml bovine serum albumin, an oxygen-scavenging system (0.22 mg/ml glucose oxidase, 0.036 mg/ml catalase, 4.5 mg/ml glucose), 127 mM non-labeled phalloidin, and the ATP-regenerating system (1 mM phosphocreatine and 0.1 mg/ml creatine phosphokinase) was infused. KCl concentration in the assay buffer was either 30 mM or increased to 100 mM (in the experiments with rabbit skeletal actins) or 150 mM (Dictyostelium actins). In case of sub-actin, at 100 mM KCl, the bead-actin attachment events were extremely rare. Therefore, in the case of skeletal wild-type actin, the movement on only long (>10-μm) filaments was analyzed, whereas in case of sub-actin, the movement on short (<10-μm) filaments was also used in the analysis. On the other hand, in the experiments with Dictyostelium actins at 150 mM KCl, the bead-actin attachment events were rare even on the Dictyostelium wild-type actin; therefore, the movement on all filaments was analyzed.

The measurements were performed at 25 ± 1 °C by the total internal reflection fluorescence microscopy (IX-71, Olympus (Tokyo, Japan)). The fluorescence images were recorded at the video rate using a digital video recorder (DSR-20, Sony (Tokyo, Japan)), and the bead movement was analyzed with the ImageJ software. To correct the 0.917 pixel aspect ratio of digital video images, which was directly determined from the image of an objective micrometer (0.01 mm) (Olympus), the images with the 720 × 480 resolution were resized to 720 × 523 for the isometric analysis. For the analysis of run lengths, all bead movement events longer than 0.5 μm (equal to 7 pixels) and 0.5 s (15 frames) during 3 min of observation were collected. For the correct measurement of run lengths on curved actin filaments, the distance from the landing point to the detachment point was determined using the Straighten plug-in. For the
Measurement of velocity, the bead movement with long run lengths (>1.1 μm, or 14 pixels) was analyzed using the Manual Tracking plug-in.

**Measurement of the Actin-activated ATPase of MMV-HMM**—Actin-activated ATPase of MMV-HMM was determined by monitoring the absorbance of NADH at 340 nm in the solution containing 56 nM MMV-HMM, 2.35 μM calmodulin, 20 mM imidazole-HCl (pH 7.4), 4 mM MgCl₂, 40 mM KCl, 1 mM EGTA, 53 μM phalloidin, 400 μM NADH (Wako (Osaka, Japan)), 800 μM phosphoenolpyruvate (Wako), 47 μg/ml pyruvate kinase (Oriental Yeast (Tokyo, Japan)), 12 μg/ml lactate dehydrogenase (Oriental Yeast), 1 mM ATP, 1 mM dithiothreitol, and various concentrations of actin (2.2–39.7 μM). The measurements were performed at 25 °C. Kₘ and Vₘₐₓ values were determined for each actin by fitting 5–9 points by Michaelis-Menten kinetics.

**Cosedimentation Assay**—The apparent affinity of MMV-S1 for actin was determined by cosedimentation assay, performed in the solution containing 20 mM imidazole-HCl (pH 7.4), 4 mM MgCl₂, 1 mM EGTA, 53 μM phalloidin, 1 mM dithiothreitol, and various concentrations of KCl (40–150 mM), as follows. First, phalloidin-stabilized F-actin (final concentration, 1 μM) was mixed with MMV-S1 (final concentration, 0.17 or 0.23 μM), supplemented with the excess calmodulin (final concentration, 2.35 μM), and incubated at 25 °C for 3 min, followed by an incubation at 4 °C for 1 min. Next, the reaction mixture (400 μM NADH, 800 μM phosphoenolpyruvate, 47 μg/ml pyruvate kinase, 12 μg/ml lactate dehydrogenase, 1 mM ATP) was added, and the sample was immediately centrifuged at 541,000 × g for 20 min at 4 °C. The supernatant and the pellet were applied to an SDS-polyacrylamide gel, and the amount of MMV-S1 was determined by densitometry using CS analyzer software (ATTRO (Tokyo, Japan)).

**RESULTS**

**Confirmation of Actin Preparations**—Rabbit skeletal wild-type actin was fully cleaved by subtilisin producing sub-actin, which was confirmed by the presence of a ~37-kDa fragment in SDS-PAGE (data not shown), as in the previous report (5). In this work, we confirmed that the primary cleavage site was between Met⁴⁷ and Gly⁴⁸ (23); however, the second cleavage site was found to be between Met⁴⁴ and Val⁴⁵ (see supplemental material for details). Non-cleaved native actin was not detected.

In the case of Dictyostelium actins, where the E360H mutation was used as a purification tag, the two-dimensional gel electrophoresis revealed that both E360H and M47A/E360H mutated actins were separated from the Dictyostelium wild-type actin (supplemental Fig. S1). As an additional control, we ran the mixed sample of the modified and wild-type actins, purified from the same preparation, and confirmed that it resulted in the appearance of two spots, whereas in the individual actin samples, a single spot was observed. For simplicity throughout, the wild-type actins, separated from E360H and M47A/E360H actins, both are referred to as the Dictyostelium wild-type actin. In all experiments, the properties of the modified actins were compared with the wild-type actin from the same preparation.

**In Vitro Motility Assay and the Measurement of the Sliding Force**—The modifications significantly affected the velocity of actin filaments in an in vitro motility assay on myosin II-HMM. Moreover, although almost all filaments of the wild-type actin were moving continuously, a significant proportion of sub-actin filaments were moving with temporary stops, each time progressing for less than their own length; otherwise, only a part of a filament was moving. The proportion of “mobile” filaments was 44% for sub-actin and 97% for the wild-type actin. The measured gliding velocity of “mobile” filaments also was significantly lower for sub-actin (Fig. 1B and Table 1).

The effect of the M47A mutation was similar although slightly less pronounced; the proportion of “mobile” filaments was 76% for E360H actin and 47% for M47A/E360H actin, and the gliding velocity was reduced ~2-fold (Fig. 1B and Table 1). The E360H mutation had no effect on the motility; both the proportion of “mobile” filaments in the case of the Dictyostelium wild-type and E360H actins (82 and 76%, respectively) and the gliding velocity were almost the same (Fig. 1B and Table 1). Therefore, the observed decrease in the velocity of M47A/E360H actin filaments is attributable to the M47A mutation.

To characterize the effect of the D-loop modifications on the strength of the actin-myosin II interaction more directly, we performed the sliding force measurement assay. The results were consistent with an in vitro motility assay (Table 1), confirming that a decrease in gliding velocity and the proportion of “mobile” filaments was due to a weaker force generated by myosin II-HMM.

Surprisingly, in an in vitro motility assay with myosin V, contrary to the experiments with myosin II, all filaments of both the wild-type and modified actins were moving normally, even when the measurements were performed at the same ionic conditions.
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TABLE 1

Summary of the effects produced by the D-loop modifications

| Myosin | Parameter | Skeletal wild type | Sub-actin |
|--------|-----------|-------------------|-----------|
| II     | Proportion of "mobile" filaments | 97% | 44% |
| Gliding velocity (μm/s) | 4.5 ± 0.50 | 0.81 ± 0.16 |
| Sliding force (piconewtons/μm) | 3.5 ± 1.0 | 1.1 ± 0.24 |
| V     | Gliding velocity (μm/s) | 0.35 ± 0.03 | 0.48 ± 0.04 |
| Single-molecule velocity (μm/s) | 0.73 ± 0.18 | 0.73 ± 0.13 |
| No. of bead-actin attachment events | 3.2 (4.4) | 0.20 (0.92) |

Run length (μm) | 2.5 ± 0.29 | 0.65 ± 0.04 |
| K_m (μM) | 3.8 ± 0.37 | 46 ± 29 |
| V_max (μm/s) | 3.5 ± 0.09 | 2.1 ± 0.85 |

Proportion in pellet | 86% | 61% |

| Dictyostelium wild type* | E360H | M47A/E360H |
|--------------------------|-------|-----------|
| Proportion of "mobile" filaments | 82% | 79% |
| Sliding force (piconewtons/μm) | 2.1 ± 0.54 | 2.3 ± 0.69 |
| Gliding velocity (μm/s) | 0.42 ± 0.03 | 0.44 ± 0.04 |
| Single-molecule velocity (μm/s) | 0.80 ± 0.18 | 0.83 ± 0.22 |
| No. of bead-actin attachment events | 0.41 (6.8) | 0.53 (5.5) |
| Run length (μm) | 1.1 ± 0.18 | 1.1 ± 0.15 |
| K_m (μM) | 3.0 ± 0.83 | 3.6 ± 1.0 |
| V_max (μm/s) | 3.6 ± 0.21 | 3.7 ± 0.27 |
| Proportion in pellet | 90% | 81% |

| E360H | M47A/E360H |
|-------|-----------|
| Proportion of "mobile" filaments | 76% | 47% |
| Sliding force (piconewtons/μm) | 2.7 ± 1.0 | 1.3 ± 0.46 |
| Gliding velocity (μm/s) | 0.43 ± 0.04 | 0.53 ± 0.03 |
| Single-molecule velocity (μm/s) | 0.81 ± 0.20 | 0.86 ± 0.18 |
| No. of bead-actin attachment events | 0.30 (7.0) | 0.10 (3.8) |
| Run length (μm) | 0.90 ± 0.05 | 0.57 ± 0.01 |
| K_m (μM) | 3.8 ± 1.1 | 69 ± 51 |
| V_max (μm/s) | 3.8 ± 0.29 | 2.9 ± 1.5 |
| Proportion in pellet | 81% | 62% |

a Mean ± S.D.

b At 200 mM KCl

c At 100 mM KCl

d At 150 mM KCl

e At 30 mM KCl

f Mean ± S.E.

i At 40 mM KCl.

strength (25 mM KCl). A decrease in the velocity due to the D-loop modifications, observed in the case of myosin II, was not detected (Fig. 2A). Moreover, when the KCl concentration was increased, which generally accelerates the gliding velocity of actin filaments (12), it became evident that, in fact, the D-loop modifications increase the gliding velocity on myosin V (Fig. 2, A and B, and Table 1).

Single-molecule Myosin V Movement Assay—The processive movement of single myosin V molecules on immobilized actin filaments was visualized (supplemental Fig. S2) by attaching them to fluorescent beads (22, 24). The D-loop modifications apparently did not affect the velocity of myosin V movement at 30 mM KCl (Fig. 3A). However, because in an in vitro motility assay, the effect of the D-loop modifications became more prominent at higher ionic strength (Fig. 2, A and B), we also performed the single-molecule movement assay at an ionic strength that was as high as possible. At a too high ionic strength (200 mM KCl), however, the processive movement of myosin V molecules on sub-actin and the M47A/E360H actin filaments was hardly observable. Thus, the experiments were performed at 100 mM KCl (on skeletal actins) or at 150 mM KCl (on Dictyostelium actins).

An increase in the velocity due to the D-loop modifications, which had been observed in an in vitro motility assay, was not detected in the movement of single myosin V molecules (Fig. 3A and Table 1). However, the D-loop modifications significantly reduced the frequency of bead-actin attachment events (Table 1).

Furthermore, whereas the run lengths at lower ionic strength (30 mM) on the wild-type actins were too long to be measured, the run lengths at higher ionic strength decreased and could therefore be determined. The D-loop modifications drastically decreased the run lengths (4-fold on sub-actin and 3-fold on the M47A/E360H actin compared with the corresponding wild-type actins). The E360H mutation also decreased the run length; however, its effect was much smaller (only a 20% decrease). Therefore, the modifications of the D-loop significantly shorten the run length of myosin V molecules. The inclusion of the movement on short actin filaments in the analysis might have affected the measured run lengths due to the presence of the molecules reaching the filament’s end. However, the proportion of such molecules was the same on the skeletal wild-type and sub-actin filaments (19 and 20%, respectively), and it was even smaller on M47A/E360H actin (8%) than on the Dictyostelium wild-type actin (29%). The E360H mutation did not affect the proportion of myosin V molecules reaching the filament end (18% on both E360H and Dictyostelium wild-type actins). Therefore, the decreased run lengths did not result from the inclusion of data collected on short actin filaments. Note that neither of the modifications noticeably decreased the length of the filaments compared with the corresponding wild-type actins.

Gliding Movement on Low Densities of Myosin V—Although the D-loop modifications increased the velocity in an in vitro motility assay on multiple myosin V molecules, the single myosin V molecules moved with the same speed on both the wild-type and the modified actins. To help resolve this paradox, we tested the effect of myosin concentration by performing an in vitro motility assay at two myosin V densities (52 or 417 molecules/μm²), using skeletal wild-type actin. The velocity slightly increased with a decrease in the density of myosin V (supplemental Fig. S3). At a too low density (<52 molecules/μm²), actin filaments were partially detached from the glass surface and did not move smoothly, so the velocity could not be reliably determined. We also note that in a previous report, the velocity of actin filaments was found to be independent of the density of myosin V (20). This may possibly be explained by the fact that those experiments were performed only at low ionic strength, which made difficult the detection of the difference in velocity (supplemental Fig. S3).

Actin-activated ATPase—The effect of the D-loop modifications on the activation of the ATPase kinetics of myosin V was determined using the recombinant MMV-HMM (Table 1). The basal ATPase rate of MMV-HMM (0.10 s⁻¹/μM) was greatly accelerated by all actins. K_m values were significantly increased by both subtilisin cleavage and the M47A/E360H mutation but remained almost unaffected by the E360H mutation (Table 1), indicating that the D-loop modifications
produce an increase in $K_m$ values. $V_{\text{max}}$ values appeared to be slightly reduced by the D-loop modifications (Table 1); however, because the velocity did not decrease in the single-molecule myosin V movement assay and even slightly increased in an in vitro motility assay on myosin V at various KCl concentrations. The velocity of the modified and the corresponding wild-type actin filaments is shown by colored and black bars, respectively. The numbers show the average velocity (μm/s). For drawing each distribution, 61–67 filaments were analyzed. The experiments were performed at four different KCl concentrations (25, 50, 100, and 200 mM). B, dependence of the velocity of actin filaments driven by myosin V on KCl concentration. The velocity is shown by black squares and solid lines (wild-type actins) or by colored squares and dashed lines (modified actins). Error bars, S.D. The ratio of the velocities at each KCl concentration is shown near each point.

**DISCUSSION**

Comparison of the Effects of Subtilisin Cleavage and the M47A Mutation—In this study, we examined the effect of the D-loop modifications on various parameters characterizing actin-myosin interaction. The D-loop is highly conserved between different classes of actin, and its sequence in rabbit skeletal and Dictyostelium actins is identical with the only exception that Gln41 of rabbit skeletal actin is replaced with Thr. Therefore, the effects of subtilisin cleavage and the M47A mutation can be directly compared even when studied with different actin classes, and the obtained results should also be applicable for the cytoplasmic actin on which myosin V actually travels in cells.

The M47A mutation, which is the replacement of a single amino acid, much less significantly affects the structure of the D-loop and, therefore, should produce a weaker effect on the interaction with myosin. The results summarized in Table 1 confirm that the effect of the D-loop modifications on various motile properties of both myosins II and V is indeed more pronounced in the subtilisin-cleaved actin compared with the M47A mutant actin. Moreover, the similarity of the trends, in which the motile properties are affected by both modifications, suggests that although in the cleaved actin the effects of the modification may propagate to the more distant elements of actin structure (25), in the experiments described here, the contribution of these allosteric effects to the interaction with myosin is negligible, if present at all, compared with the impact of the modifications within the D-loop itself.

**Myosin II Generates Weaker Force on Modified Actins**—The modifications of the D-loop markedly decreased the gliding velocity of actin filaments on myosin II-HMM (Fig. 1B), which...
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Effects of the Modifications on the Motility of Myosin V—A single-molecule movement assay of myosin V showed that the D-loop modifications markedly decreased both the run lengths and the frequency of the bead-actin attachment events, although the velocity was not affected (Fig. 3A and Table 1). These results are very similar to the experiments with the recombinant MMV-HMM, in which the positive charge of loop 2 was reduced (26). Surprisingly, although the D-loop modifications increased the velocity of actin filaments in an in vitro motility assay (Fig. 2, A and B), individual myosin V molecules moved with the same speed on both the wild-type and modified actins, which was much faster than the gliding velocity of actin filaments of the same type on multiple myosin V molecules. We explain this result by the internal friction, which multiple myosin molecules impose on each other by simultaneously binding the same actin filament. In the case of myosin II, the cooperative drive by multiple myosin molecules is necessary to continuously generate force on an actin filament, because the lifetime of the strongly actin-bound states is very short. On the contrary, myosin V molecules remain attached to actin for a long time. Therefore, in an in vitro motility assay on myosin V, the non-coordinated multiple motors impose internal friction on each other, which slows down actin filaments. An increase in the velocity of the modified actins is therefore induced by a decrease in the number of the myosin molecules attached to the same actin filament at the same time, which correlates with the shorter run lengths of single myosin V molecules on the D-loop-modified actins and with both an increase in $K_m$ values determined by measuring the actin-activated ATPase and the lower affinity observed in the cosedimentation assay, produced by the D-loop modifications (Fig. 3B and Table 1).

An in vitro motility assay performed at lower myosin densities supports this conclusion. An increase in the gliding velocity with a decrease in the density of myosin V was at most 20% (supplemental Fig. S3), whereas the observed acceleration of the velocity on sub-actin was as much as 37% (Fig. 2B, right). Therefore, the observed increase in the velocity of the D-loop-modified actin filaments cannot be sufficiently accounted for only by a smaller number of the simultaneously interacting myosin V molecules compared with the wild-type actin, and we conclude that the D-loop modifications do increase the gliding velocity of actin filaments by decreasing myosin’s affinity for actin and, consequently, reducing the internal friction generated by the strongly binding myosin V molecules in the ADP-bound state.

Step in the ATPase Cycle of Actomyosin in Which the D-loop Is Involved—The coupling of mechanical and chemical cycles in myosin V has been extensively studied, and the general consensus has been achieved, which allows us to make reliable assumptions about which step in the ATPase cycle is affected when the variations in motility are observed. According to the “hand-over-hand” model (27, 28), the unbound head of myosin V lands on an actin filament in the ADP-P$_i$ state. If the modifications of the D-loop weaken the actin-binding affinity of myosin V in the ADP-P$_i$ state, this would slow down the transition of the leading head from the ADP-P$_i$ state to the strongly bound ADP state. This would increase the possibility of a single-headed binding to actin by only the trailing head and, accord-
ingly, dissociation from actin, resulting in a shorter run length without affecting the velocity of the myosin V stepping (see below), which correlates with the results obtained in this study. At the same time, the D-loop modifications weaken the force generation by myosin II, which has been predicted to occur in the myosin-ADP state (29). Therefore, the D-loop is involved in actin-myosin interaction most plausibly in the actomyosin-ADP-P state, state of both myosins II and V. This conclusion is supported by the results of Volkmann et al. (30), who reported that loop 2 of myosin V in the ADP-P state interacts with subdomain 2 of actin, which contains the D-loop, whereas the probability of the interaction in the myosin-ADP state is smaller.

However, the possibility of the D-loop contributing to binding in the myosin-ADP state should also be considered. According to the “hand-over-hand” model, the trailing head of myosin V is in the ADP-bound or the nucleotide-free state. At 1 mM ATP used in our experiments, the contribution of the nucleotide-free state can be ruled out, because ATP rapidly binds to the nucleotide-free head (31). If the modifications facilitate the detachment of the ADP-bound trailing head from actin, the run length should decrease, because the trailing head can detach prior to the transition of the leading head from the weakly binding ADP-P state to the ADP state. The involvement of the ADP state is, however, less plausible than the contribution of the D-loop to binding myosin in the ADP-P state, because ADP release from the trailing head is the rate-limiting step in the processive stepping of myosin V (31, 32), and its alteration would have affected the velocity of the myosin V stepping, which was not observed.

The cosedimentation assay also supports this conclusion. The apparent affinity of the D-loop-modified actin for MMV-S1 in the presence of ATP decreased compared with the wild-type actin, whereas in the presence of ADP, a decrease in the affinity has not been detected (supplemental Fig. S4B), indicating that the D-loop modifications decrease the myosin’s affinity for actin when the motor is in the ADP-P state.

Our results reveal that the conformational changes in the D-loop affect the motile properties of myosin, regardless of the myosin class. This observation indicates that the conformational changes in the D-loop can control the motility and, therefore, the intracellular performance of myosins. Myosin V transports organelles along actin filaments; however, it remains unclear how the motor recognizes the starting and the ending points of an organelle-transporting run. The obtained results suggest that the D-loop is involved in actin-myosin interaction, when myosin is in the ADP-P state. Considering that the unbound head of myosin V lands on actin with ADP-P, in its active site, the affinity for actin in this state is crucial to avoid premature termination of the processive run due to the dissociation of the trailing head prior to the leading head strongly binding actin. The conformational changes in the D-loop may thus control myosin V affinity for actin and the length of the processive runs. Our results therefore suggest a model in which the D-loop is involved in the local regulation of the organelle transport by myosin V (Fig. 4) and controls force generation by myosin II. Subdomain 2 of actin, which contains the D-loop, tends to dynamically change its conformation when interacting with many kinds of binding proteins (e.g. myosin (4, 13) and cofilin)

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FIGURE 4. Model of how the D-loop may regulate the organelle transport. Gray spheres indicate the actin subunits, in which the D-loop increases the affinity of myosin V-ADP-P for actin (high affinity D-loop). Red spheres indicate the actin subunits in which the affinity for myosin V-ADP-P is decreased by the D-loop conformation (low affinity D-loop). Myosin V tends to bind to actin in the high affinity D-loop state and run long distances until it enters the low affinity D-loop area, where myosin does not bind strongly and tends to detach. Thus, the high affinity D-loop area and the low affinity D-loop area, which may be switched between by the attachment of various actin-binding proteins, are the starting and the ending points, respectively, of an organelle-transporting run. (33)). Thus, the conformation of the D-loop may vary, depending on the local conditions in the cell and control the cellular functions of myosin.

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