Mutational Analysis of the Switch II Loop of Dictyostelium Myosin II*

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A loop comprising residues 454–459 of Dictyostelium myosin II is structurally and functionally equivalent to the switch II loop of the G-protein family. The consensus sequence of the “switch II loop” of the myosin family is DIXGFE. In order to determine the functions of each of the conserved residues, alanine scanning mutagenesis was carried out on the Dictyostelium myosin II heavy chain gene. Examination of in vivo and in vitro motor functions of the mutant myosins revealed that the I455A and S456A mutants retained those functions, whereas the D454A, G457A, F458A and E459A mutants lost them. Biochemical analysis of the latter myosins showed that the G457A and E459A mutants lost the basal ATPase activity by blocking of the isomerization and hydrolysis steps of the ATPase cycle, respectively. The F458A mutant, however, lost the actin-activated ATPase activity without loss of the basal ATPase activity. These results are discussed in terms of the crystal structure of the Dictyostelium myosin motor domain.

In the Dictyostelium motor domain designated as S1dC (1), a bound nucleotide is surrounded by three loops whose sequences are highly conserved among the myosin family (2): the P-loop (residues 179–186 of Dictyostelium myosin II) and the two loops in the 50K segment (residues 233–240 and 454–459 of Dictyostelium myosin II) (see Fig. 1A). One of the loops in the 50K segment (residues 233–240) is homologous to a loop in the switch I region of GTPases judging from the topological similarity (3) and has the consensus sequence NNNSRFG (NNNSSRFG in Dictyostelium myosin II). Residues in the loop are aligned along the ATPase pocket, and some of the side chains form hydrogen bonds with the bound nucleotide. The other loop in the 50K segment has the consensus sequence, DIXGFE (DISGFE in Dictyostelium myosin II) and is functionally and structurally equivalent to a loop in the switch II region of GTPases (3). In GTPases, the switch II loop connects the GTPase site and the switch II ␣-helix, which is part of the effector binding region. Information on the nucleotide state at the ATPase site is transmitted to the effector binding region partially through this switch II loop. In myosin, the switch II loop connects the ATPase pocket and a long conserved ␣-helix embedded in the lower 50K subdomain (4, 5).

Recent x-ray crystallographic studies on Dictyostelium S1dC complexed with various nucleotides and nucleotide analogs revealed that the switch II loop undergoes a significant conformational change during ATP hydrolysis (Fig. 1B) like the loop in GTPases. When S1dC is complexed with MgADP/BeFx, MgADP/AlFx (the V concentration dependence of the basal ATPase activity and the myosin-null cells in which the myosin II heavy chain gene had been knocked out by means of homologous recombination (10). Dictyostelium cells transformed by electroporation

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1 The abbreviations used are: MgATP⋅S, adenosine 5′-O-(thiotriphosphate); MOPS, 4-morpholinepropanesulfonic acid; NTA, nitrosoic acid; HPLC, high performance liquid chromatography.
Arg-238–Glu-459 interaction, the side chain of Arg-238 is shown as a switch II loop are shown as a stick model. Gly-457 are in yellow, from the BeFx structure (1) to the V i structure (right). The two residues in the core of the lower 50K subdomain. These residues, Residues in the core of the lower 50K subdomain. These residues, Asn-472, Asn-475, His-572, Tyr-573, and Ala-574, are shown as space-filled models in yellow. They form a hydrophobic pocket for Phe-458. The residues in the switch II loop are shown as a stick model in green. The bound nucleotides are shown as a space-filled model in cyan. Note that the Arg-238–Glu-459 contact is in contact with the V i moiety of MgADP/Vi, whereas it is located away from MgADP/BeFx. C, hydrophobic interactions between F458 and residues in the core of the lower 50K subdomain. These residues, Asn-472, Asn-475, His-572, Tyr-573, and Ala-574, are shown as space-filled models in yellow. They form a hydrophobic pocket for Phe-458. The residues in the switch II loop are shown as a stick model in green. The bound nucleotides are shown as a space-filled model in magenta. Note the swinging and rotation of Phe-458 and also the accompanying changes in the residues in the lower 50K subdomain on the transition from the BeFx structure (left) to the V i structure (right). The two structures are shown in orientations that allow their upper 50K subdomains to be superimposed.
Assays were carried out as described (29–31). To determine the amount of the fluorescent nucleotide released, in vitro MgATPase activities were measured as described (32).

### RESULTS

#### Cell Phenotypes Expressing Recombinant Myosins—Dictyostelium

Myosin II-null cells could not undergo normal cytokinesis and only slowly grew in suspension up to the density of ~1 × 10^6 cells/ml, becoming multinucleated cells (Fig. 2, Null). When a multicopy plasmid bearing the wild-type heavy chain gene of myosin II was introduced into myosin-null cells, the defect in cytokinesis was reversed so that the resulting transformants (designated as “wild-type cells”) could grow in suspension as mononucleated cells up to the density of ~2 × 10^7 cells/ml (Fig. 2, WT). The other transformants expressing the mutant myosins could be grouped into two types according to their behavior in suspension culture (Fig. 2). I455A and S456A cells grew like the wild-type cells, although D454A, G457A, F458A, and E459A cells did not grow or grew only slowly, like myosin-null cells. When transformed Dictyostelium cells were allowed to develop on agar plates covered with E. coli cells, the I455A and S456A cells, which grew well in suspension, formed fruiting bodies like the wild-type cells. However, the D454A, G457A, F458A, and E459A cells, which had a defect in suspension culture, could not develop beyond the mound stage, like myosin-null cells.

#### Motor Functions of the Purified Myosins—The actin-activated and basal MgATPase activities of the purified myosins were measured (Fig. 3). The D454A, G457A, F458A, and E459A myosins exhibited very low V_max values for the actin-activated ATPase activity per ATPase site. They were calculated from SV versus S plots (S is the concentration of actin filaments and V is the rate of phosphate release) for at least three independent measurements. Basal MgATPase activity per ATPase site.

In vitro motility assays were carried out as described (29–31).
myosins (Fig. 4). As expected from the phenotypes of cells expressing the mutants, the D454A, G457A, F458A, and E459A myosins could not drive the sliding of actin filaments, whereas the I455A and S456A myosins drove the sliding.

Determination of the Step Blocked by Mutations—As described above, the D454A, G457A, and E459A mutations almost completely abolished the ATPase activity. To determine which step of the ATPase cycle was blocked by the mutations, the binding of mant-ATP to these myosins was measured. Since all these myosins lost their ATPase activities, it was possible to carry out stepwise titration without appreciable hydrolysis of mant-ATP during the measurements. As shown in Fig. 5, the G457A myosin bound mant-ATP tightly, whereas the D454A and E459A myosins bound it more weakly. As shown below, however, it seems that the weak binding of mant-ATP to the E459A myosin was because of the slow binding of the fluorescent nucleotide to the mutant, not to its intrinsically low affinity. These results suggest that the D454A, G457A, and E459A myosins could bind mant-ATP with various affinities, implying that these mutations did not block the ATP binding to the myosins, but blocked the ATP hydrolysis step, like the R238A mutation in the switch I loop (32).

To further investigate the step blocked by the G457A and E459A mutations, the single turnover of the ATPase reaction was followed using a fluorescent ATP analog, Cy3-ATP. As shown in Fig. 6, the wild-type myosin showed an “ADP burst” (~0.7 mol/mol of ATPase-site), reflecting the quick ATP hydrolysis step followed by the rate-limiting P_i release step. Unlike the wild-type myosin, the G457A myosin did not show such an “ADP burst” (Fig. 6), indicating that the G457A mutation blocked the ATP hydrolysis step, not the P_i release step. Similar results were recently reported for smooth muscle myosin (33). Like the G457A myosin, the E459A myosin did not show an ADP burst (Fig. 6). This result supports, but does not necessarily confirm, that the mutation blocked the hydrolysis step, given the fact that the rate of mant-ATP binding to E459A myosin was unexpectedly slow, as shown below.

Actin-S1dC Interaction—The ATP-dependent association and dissociation of the *Dictyostelium* myosin motor domain, S1dC (1), with pyrene-labeled F-actin was studied, the pyrene fluorescence being followed (28). S1dC was used mainly because more reproducible data were obtained using this soluble, single-headed fragment. As previously shown, the pyrene fluorescence decreased when the wild-type S1dC formed a rigor complex with the pyrene-labeled actin (34). On the addition of ATP, S1dC was transiently dissociated from the F-actin, and then reassociated with it after ATP had been completely hydrolyzed to ADP and P_i. Thus, the pyrene fluorescence transiently increased and then decreased (Fig. 7A). F458A S1dC also formed a rigor complex with F-actin in the absence of ATP, as indicated by the decrease in the pyrene fluorescence (Fig. 7B). The rigor complex was transiently dissociated on the addition of ATP and was formed again after ATP had been exhausted. Complete dissociation of the rigor complex was achieved only when a large excess of ATP was added because F458A S1dC retained high basal ATPase activity and, therefore, quickly consumed ATP. Thus, F458A S1dC exhibited normal ATP-dependent dissociation-association with F-actin even though it had lost its actin-activated ATPase activity. When G457A S1dC was mixed with the pyrene-labeled F-actin in the absence of ATP, a rigor complex was formed, as judged from the decrease in the pyrene fluorescence. On the addition of a small amount of ATP (even 1 mol/mol of S1dC), almost complete dissociation of the rigor complex occurred (Fig. 7C), indicating that G457A S1dC entered in a weak-binding state when it bound ATP. Because of the lack of ATPase activity, G457A S1dC remained in this weak-binding state. Unlike these mutants, however, E459A S1dC, purified by either the standard or the alternative procedure (see “Experimental Procedures”), failed to form a rigor complex with F-actin even in the absence of ATP, as judged from the fact that the pyrene fluorescence of F-actin never decreased on the addition of the purified S1dC.

Tryptophan Fluorescence—The intrinsic tryptophan fluorescence of the wild-type S1dC increased on the addition of excess ATP (Fig. 8A), as previously reported for a similar fragment of *Dictyostelium* myosin (34). On the addition of MgADP, a slight decrease in the fluorescence intensity was observed. Unlike that of the wild-type S1dC, however, the tryptophan fluores-
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Random Mutagenesis of the 459th Residue—The 459th residue was randomly mutagenized using the E459A myosin gene as a template. Then the mixture of mutagenized myosin genes was introduced into myosin-null cells. When transformed cells were allowed to grow in the presence of E. coli cells, they formed two types of plaques that were easily distinguishable from their diameters. The diameter of the larger plaques was 2-fold larger than that of the smaller ones, which was almost the same as that of myosin-null cells. Among the 672 plaques isolated on the transformation of 10⁷ cells, 36 plaques were of the larger type. The diameter of a plaque is a good indicator of the in vitro motor functions of myosin (20, 24).

DISCUSSION

Mutant myosins generated by alanine scanning mutagenesis of the switch II loop can be classified into two groups according to their in vivo phenotypes. One group, comprising the I455A and S456A myosins, fully reverse the myosin-specific defects of myosin-null cells. The other group, comprising the D454A, G457A, F458A, and E459A myosins do not reverse any of the defects.

Although I455 is a highly conserved residue in almost all myosins (2) and functions as a pivoting residue for the main chain rotation of the switch II loop during the transition from the V₅ structure to the BeFx structure (5, 6), the I455A myosin retained most of its motor functions. It must be noted that when the other pivoting residue, G457, was replaced with alanine, the motor functions were completely lost. It seems that the I455A mutation did not block the main chain rotation of the switch II loop, unlike the G457A mutation. Another mutant in the first group, the S456A myosin, exhibited normal motor functions, as expected from the facts that this residue is not conserved among myosins and that alanine occupies this position in some myosins (2).
Fig. 8. Tryptophan fluorescence of the wild-type and mutant S1dC. A, the wild-type S1dC in the absence (solid line) and presence (dotted line) of MgATP or MgADP. B, G457A S1dC (solid line) and E459A S1dC (dotted line). Their tryptophan fluorescence did not change on the addition of MgATP or MgADP. C, F458A S1dC in the absence (solid line) and presence (dotted line) of MgATP or MgADP. S1dC, 1 μM; ATP or ADP, 0.1 mM. Excitation, 290 nm. Tryptophan fluorescence spectra of wild-type, G457A, and E459A S1dC were normalized by those of completely denatured proteins in 6 M GuHCl.

Incorporation and release of mant-ATP. A, incorporation of mant-ATP to E459A S1dC. E459A S1dC (1 μM) and mant-ATP (20 μM) were mixed in 50 mM NaCl, 10 mM MOPS, pH 7.4, and 2 mM MgCl2. The mixture was passed through a HPLC gel filtration column to determine the amount of the released mant-ATP. The mixture was passed through a HPLC column to determine the amount of the bound fluorescent nucleotide. B, release of the trapped mant-ATP from the E459A S1dC-mant-ATP complex. The complex was purified after incubating the above mixture for 2 days. At various times after purification, it was passed through the HPLC column to determine the amount of the released mant-ATP.

Fig. 9. Incorporation and release of mant-ATP. A, incorporation of mant-ATP to E459A S1dC. E459A S1dC (1 μM) and mant-ATP (20 μM) were mixed in 50 mM NaCl, 10 mM MOPS, pH 7.4, and 2 mM MgCl2. The mixture was passed through a HPLC gel filtration column to determine the amount of the released mant-ATP. B, release of the trapped mant-ATP from the E459A S1dC-mant-ATP complex. The mixture was purified after incubating the above mixture for 2 days. At various times after purification, it was passed through the HPLC column to determine the amount of the released mant-ATP.

The position of Gly-457 relative to the γ-phosphate of ATP in the ATPase pocket may change on rotation of the main chain of the switch II loop, depending on the state of the nucleotide. Thus, Gly-457 in Dictostelium myosin seems to function like the “γ-phosphate sensor” glycine in GTPases (for example, Gly-60 in Ras). It is likely that the G457A mutation blocked this rotation of the main chain because of steric hindrance. Given the fact that the G457A mutant was trapped before the isomerization step when it bound ATP, it is tempting to speculate that the isomerization step is coupled with the rotation of the main chain of the switch II loop (33), which occurs on the transition from the BeFx structure to the V structure, and that G457A S1dC-ATP takes on the BeFx structure. Consistent with this notion, mant-ADP/BeFx was trapped in G457A S1dC, whereas mant-ADP/BeFx was not (data not shown).

The side chain of Glu-459 is located close to the bound nucleotide in the V structure of Dictostelium S1dC, forming a hydrogen bond with a water molecule suitably positioned to participate in ATP hydrolysis (6). This strategic location of Glu-459 suggests that the residue is crucial for the hydrolysis step. In fact, the E459A mutant was unable to hydrolyze ATP because the ATP hydrolysis step was blocked (Fig. 10). Once ATP was in the ATPase pocket of the E459A mutant, it was almost irreversibly trapped there without hydrolysis, as observed here. The results suggest that the E459A mutant was trapped possibly at the M*•ATP state (Fig. 10) (35). Besides its role in ATP hydrolysis, Glu-459 may also play a role as a “gatekeeper” of the backdoor for P1 release (8), opening and closing it through the ionic interaction with Arg-238 (Fig. 1B) (32). This notion implies that ATP hydrolysis is tightly coupled with the opening and closing of the backdoor. The crucial importance of Glu-459 was also highlighted by the observation that the motor functions were retained only when glutamic acid occupied the 459th position.

The side chain of Asp-454 faces the ATPase pocket, and is coordinated to an Mg ion of the bound nucleotide through a water molecule (5, 6). Ser-237 in the switch I loop of Dictostelium myosin is also directly coordinated to the Mg ion from the other side of the ATPase pocket. Unlike the S237A myosin (32), however, the D454A myosin bound mant-ATP, although weakly, indicating that Asp-454 is of secondary importance in retaining the MgATP in the ATPase pocket, whereas Ser-237 is essential for this.

In contrast to the D454A, G457A, and E459A myosins, the F458A myosin in the second group retained the basal MgATPase activity although it completely lost the actin-activated ATPase activity. The observed in vivo defects of the F458A myosin arose from the lack of this essential ability to power the motor. The side chain of Phe-458 points away from the ATPase pocket and is buried in a hydrophobic pocket formed by residues such as Asn-472, Asn-475, His-572, Tyr-573, and Ala-574 in the core of the lower 50K subdomain (5). Therefore, disruption of the hydrophobic interaction by the F458A mutation blocks some of the structural changes expected to occur during the ATPase cycle, forcing the mutant to bypass some intermediate states. In fact, in the presence of ATP, F458A S1dC was in a unique steady state quite different...
from M**-ADP-Pi, as judged from the tryptophan fluorescence intensity. The F458A mutant in this unique steady state failed to interact with F-actin in such a way that it stimulated the actin-activated ATPase activity. Further kinetic and structural studies on the F458A myosin would reveal how F-actin triggers the actin-activated ATPase activity.

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