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Two Functional Heads Are Required for Full Activation of Smooth Muscle Myosin*

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The motor activity of smooth muscle myosin II is regulated by the regulatory light chain phosphorylation, but it is not understood how phosphorylation activates motor activity. To address this question, we produced asymmetric heavy meromyosin (HMM), which is composed of a wild-type (WT) heavy chain and a mutant heavy chain having no motor activity (*i.e.* S236T or G457A). The actin-activated ATPase activities (V_{max}) of asymmetric HMMs were only 21.8 and 8.4% of the wild-type HMM for S236A/WT HMM and G456A/WT HMM, respectively. If the two heads of HMM are independent for their ATPase activities, asymmetric HMM should show 50% of the activity of wild-type HMM; however, the activity of asymmetric HMM was much lower than the expected value. The results suggest that the activity of the wild-type head is attenuated by the presence of inactive head. Consistently, the actin-gliding velocity of the asymmetric HMM (*i.e.* S236T/WT or G457A/WT) was less than one-fifth of the wild-type HMM. The present study supports an idea that the two heads of smooth muscle myosin II interact with each other and the presence of two active heads is required for full activation.

Myosin II is a hexametric molecule composed of two heavy chains, two essential light chains (ELC),[1] and two regulatory lights chains (RLC). Each heavy chain consists of a globular motor domain, and a light chain binding domain, and a α-helical coiled-coil rod (1–4). It is known that the motor activity of smooth muscle, and nonmuscle myosin II is activated by phosphorylation of RLC. The dephosphorylated forms of those myosins have little actin-dependent ATPase activity and are unable to move actin filaments *in vitro*, whereas the phosphorylated forms are active in both ATPase and motility activities. The location of RLC on myosin molecule is at the C-terminal long α-helix portion of the heavy chain in the myosin head region (5), and the phosphorylation site, Ser-19 on RLC, responsible for the activation of myosin motor activity (2–4), is far from the effector sites (*i.e.* ATP and actin binding sites in the motor domain). Therefore, it is unlikely that the phosphate moiety on RLC directly interacts with the effector sites, thus activating the motor activity of myosin II. This raises a hypothesis that the phosphorylation at Ser-19 on RLC induces a conformational change in the head of myosin II, and this facilitates the product release from the active site upon actin binding, thus activating the motor activity. A key question is whether the nature of such a conformational change is responsible for the activation of the motor function. It has been known that the phosphorylation-dependent regulation of the myosin II motor activity requires the two-headed structure of myosin II (6–10). A key component of the proposed mechanism is that the RLC phosphorylation alters the interaction between the two heads of myosin II that converts the inhibited form to the activated form.

However, the nature of such an interaction between the two heads, which are required for the full activation, is still unclear. Previously, we produced SMDHMM, a single-headed HMM, which is composed of one intact head and one truncated head having an RLC domain and the coiled-coil tail and found that whereas dephosphorylated SMDHMM has little actin-activated ATPase activity, the phosphorylated SMDHMM is partially activated, suggesting that the interaction between the two RLC domains on the two heads is important but not sufficient for the full activation (11). Sweeney *et al.* (12) further showed that even the presence of the entire light chain binding domain is not sufficient for the full activation of myosin II by phosphorylation, implying that full regulation of ATPase activity and motility can only be obtained with two intact heads. Recently, Konishi *et al.* (13) reported that phosphorylated smooth muscle HMM has much higher actin-activated ATPase activity than the phosphorylated single-headed smooth HMM and smooth myosin subfragment 1. These findings suggest that an entire head is required for the phosphorylation-induced full activation of the motor function of myosin II. A critical question is whether the structure of the entire head itself is sufficient for the activation. In other words, does the activity of the one head influence the activity of the other head?

In the present study, we produced asymmetric smooth muscle HMMs that are composed of a wild-type heavy chain and a mutant heavy chain, in which the motor activity is inactivated by mutation. We produced two mutant heavy chains, in which Ser-236[2] and Gly-457 are changed to Thr and Ala, respectively. It was shown previously that the mutation of Ser-236 in the Switch I loop to Thr completely abolished the actin-activated ATPase activity, although the mutant can produce the ternary complex (myosin-ADP-P) with a normal rate constant (14). On the other hand, the mutation of Gly-457 in the Switch II loop to Ala abolishes the ATP hydrolysis of myosin II, thus failing to form the ternary complex, although it can bind ATP and dis-

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[1] The abbreviations used are: ELC, essential light chain; HMM, smooth muscle heavy meromyosin(s); RLC, regulatory light chain; DTT, dithiothreitol; MLCK, myosin light chain kinase; WT, wild-type; ATPγS, adenosine 5′-*O*-(thiotriphosphate); Mant, 2-(3)-*O*-(*N*-methylanthraniloyl).

[2] We have used sequence numbers for *Dictyostelium* myosin II for consistency with our previous reports and ease in comparison with the structural studies of the active site. The corresponding gizzard smooth muscle myosin sequence numbers (in parentheses) are Ser-236 (244) and Gly-457 (468).

sociate from actin (15). We isolated HMMs having a mutant and a wild-type head and characterized their motor activities.

Our results clearly showed that the presence of two functional myosin heads is required for the full activation of smooth myosin by phosphorylation, implying the cooperativity between two phosphorylated heads.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (16). Smooth muscle myosin light chain kinase (MLCK) was prepared from frozen turkey gizzard (17). Recombinant calmodulin of *Xenopus* oocyte (18) was expressed in *Escherichia coli* as described (19). Ni^{2+}-nitrilotriacetic acid-agarose was purchased from Qiagen (Hilden, Germany). Anti-FLAG M2 antibody and anti-FLAG M2 affinity gel were from Sigma. FLAG peptide (Tyr-Lys-Asp-Asp-Asp-Asp-Lys-His) was synthesized in the Peptide Core Facility at the University of Massachusetts Medical School. Anti-His antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mant-2-dATP was provided by Dr. Howard D. White (Eastern Virginia Medical School).

Construction of Smooth HMM Mutants—A smooth muscle HMM (6D3) in baculovirus transfer vector pBluebac4 was used as template for the following manipulation. A His tag (His_6) or FLAG tag (Tyr-Lys-Asp-Asp-Asp-Asp-Lys-His) was created at the 3′ side of the smooth HMM heavy chain (6D3) by site-directed mutagenesis. Those two constructs were named 6D3His and 6D3Flag. Two *Spe*I sites were created at the 5′ side of the initiation codon and the 3′ side of the stop codon by site-directed mutagenesis. 6D3His or 6D3Flag having *Spe*I sites were digested by *Spe*I and ligated into pFast-*Nhe*I(+) transfer vector (which was derived from the original pFastBac transfer vector by introducing *Nhe*I in the polylinker region). Two single site mutations, S236T and G457A, were introduced by site-directed mutagenesis by using 6D3His/pFast as templates as described previously (14). The mutations were confirmed by direct sequencing analysis. The baculovirus for expressing HMM heavy chain was prepared by using the Bac-N-Blue kit (Invitrogen). The recombinant baculoviruses expressing ELC or RLC were prepared as described before (8).

Expression and Purification of Asymmetric HMM—To express WT(F)/ST(H) (asymmetric HMM-S236T, one wild-type head and one S236T mutant head) (Fig. 1), 200 ml of sf9 insect cells, co-infected with four separate viruses encoding 6D3-WT-Flag, 6D3-S236T-His, ELC, and RLC, were cultured in a spinner flask containing 600 ml of TMN-FH medium (Sigma) for 3 days. After the cells were harvested by centrifugation, the cell pellets were lysed with 30 ml of Lysis Buffer (0.4 M KCl, 50 mM KP_i, pH 7.5, 15 mM imidazole, 5 mM $MgCl_2$, 0.2 mM EGTA, 5 mM β-mercaptoethanol, 2 mM phenylmethanesulfonyl fluoride, 0.01 mg/ml leupeptin, 1 mg/ml trypsin inhibitor, 0.5% Triton X-100, and 5 mM ATP) with sonication. All of the purification procedures were performed at 4 °C unless otherwise indicated. After centrifugation at 120,000 × *g* for 30 min, the supernatant was incubated with 2 ml of Ni^{2+}-agarose in a 50-ml conical tube on a rotating wheel for 1–2 h. The resin suspension was then loaded on a column (1 × 10 cm) and was washed with 30 ml of solution A (50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.1 mM EGTA, and 1 μg/ml leupeptin). WT(F)/ST(H) and ST(H)/ST(H), bound to Ni^{2+}-agarose, were eluted with solution B (0.2 M imidazole pH 7.5, 0.2 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 μg/ml leupeptin), and 1 ml of each fraction was collected. The fractions containing protein were pooled and loaded on a column containing 1 ml of anti-FLAG M2 affinity gel pre-equilibrated with solution A. After washing with 10 ml of solution A, the column was eluted with 0.2 mg/ml FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) in solution A. Only WT(F)/ST(H) bound to the anti-FLAG M2-agarose gel and was eluted by FLAG peptide.

Similar procedures as above were used for expression and preparation of WT(F)/GA(H) (asymmetric HMM-G457A with one wild-type head and one G457A mutant head) and WT(F)/WT(H), except 6D3-G456A-His or 6D3-WT-His was used instead of 6D3-S236T-His for co-infection of sf9 cells. To express WT(H)/WT(H) or WT(F)/WT(F), which has only one kind of tag (His tag or FLAG tag, respectively), sf9 cells were co-infected with three kinds of baculoviruses expressing 6D3-WT-His or 6D3-WT-Flag, ELC, and RLC. Wild-type homodimer HMM (*i.e.* WT(H)/WT(H) or WT(F)/WT(F)) was purified by a single affinity chromatography, Ni^{2+}-agarose, or anti-FLAG M2 affinity gel, respectively. Mutant homodimer HMM (*i.e.* ST(H)/ST(H) or GA(H)/GA(H)) was produced by co-infection of three kinds of baculoviruses expressing 6D3-S236T-His or 6D3-G457A-His, ELC, and RLC, and prepared by Ni^{2+}-agarose chromatography. Approximately 0.1 mg of the asymmetric molecules and 1 mg of symmetric molecules were purified from 600 ml of culture.

Phosphorylated HMMs were prepared as following. About 1 μM of purified HMM was incubated with 10 μg/ml MLCK, 15 μg/ml calmodulin, and 0.5 mM ATPγS in a solution of 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM ATPγS, and 5 mM mercaptoethanol at 25 °C for 30 min. Phosphorylated HMM was repurified by Ni^{2+}-agarose chromatography. The phosphorylation of RLC was confirmed by urea gel (20).

All of the above samples were dialyzed extensively with solution D (20 mM Tris-HCl, pH 7.5, 48 mM KCl, 1 mM DTT) overnight. The dialyzed samples were clarified by centrifugation at 15,000 rpm for 5 min. The concentration of purified HMM was determined by the Bradford method (21) using chicken gizzard smooth muscle HMM as a standard.

Single Turnover ATPase Assay—This assay measures the rate of phosphate release from the acto-HMM ADP·P_i state. Since phosphate release is rate-limiting, the decrease in fluorescence as Mant ADP dissociates from HMM can be used to measure phosphate release rates. Assays were performed at 25 °C in a temperature-controlled stopped-flow spectrophotometer (KinTek Co., Clarence, PA) equipped with a 75-watt xenon lamp. Mant nucleotide was excited at 360 nm, and emission was collected through a 420-nm cut-off filter. The experiment was done with a three-syringe system for double mixing. The first syringe (1 ml) contained 0.5 μM thiophosphorylated myosin head (WT(F)/WT(H) or WT(F)/GA(H)) in a solution containing 20 mM Tris-HCl, pH 7.5, 48 mM KCl, 2 mM, and 1 mM DTT; the second one (1 ml) contained 1 μM Mant-2-dATP in a solution of 20 mM Tris-HCl pH 7.5 and 4 mM $MgCl_2$; and the third one (1 ml) contained 0 or 40 μM (equal to 1.74 mg/ml) of actin in a solution of 20 mM Tris-HCl, pH 7.5, 24 mM KCl, 2 mM $MgCl_2$ and 1 mM ATP. The delay time between the first and second mixing was 5 s. The conditions for first mixing were as follows: 0.25 μM myosin head, 0.5 μM Mant-2-dATP, 24 mM KCl, 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 0.5 mM DTT. The conditions for the second mixing were as follows: 0.167 μM myosin head, 0.333 μM Mant nucleotide, 0 or 13.3 μM (equal to 0.58 mg/ml) actin, 333 μM ATP, 24 mM KCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, and 0.333 mM DTT.

The collected data were analyzed with the Kaleidagraph software (Synergy Software, Reading, PA). Data were fit to a double exponential model, because a single exponential model consistently missed a fast phase for both samples.

Other Assays—Steady state ATPase assay, *in vitro* actin-gliding assay, and SDS-PAGE are the same as described previously (14).

RESULTS

Expression and Purification of Asymmetric HMM—To produce asymmetric HMM, we introduced the two distinct tags, a His tag and a FLAG tag, at the C-terminal end of the smooth muscle HMM constructs, respectively. The sf9 cells were co-infected with recombinant baculoviruses expressing HMM heavy chain with His tag, HMM heavy chain with FLAG tag, ELC, and RLC, respectively, to prepare HMM composed of a heavy chain with a His tag and a heavy chain with a FLAG tag. First, we prepared the wild-type HMM containing a His tag and a FLAG tag on each heavy chain. When the cells were co-infected with the recombinant baculoviruses expressing the heavy chain, ELC, and RLC, respectively, three kinds of HMMs, WT(H)/WT(H), WT(F)/WT(H), and WT(F)/WT(F), were produced (see Fig. 1). To isolate the heterodimer, WT(F)/WT(H), from the two types of homodimers (*i.e.* WT(H)/WT(H) and WT(F)/WT(F)), the cell extract was subjected to two types of affinity chromatography, Ni^{2+}-agarose affinity chromatography, and anti-FLAG M2 affinity chromatography. The extract was applied to a Ni^{2+}-agarose column, and HMM containing His tag was eluted by 0.2 M imidazole-containing buffer. The eluted fraction contained WT(H)/WT(H), a homodimer of HMM containing His tag on both heavy chains, and WT(H)/WT(F), a heterodimer containing His tag and FLAG tag on each of the heavy chains. The mixture was then applied to an anti-FLAG M2 affinity column, and HMM containing a FLAG tag was eluted by adding FLAG peptide. As a control, the homodimers HMM containing two His-tagged heavy chains (*i.e.* WT(H)/WT(H), ST(H)/ST(H), or GA(H)/GA(H)) and two FLAG-tagged

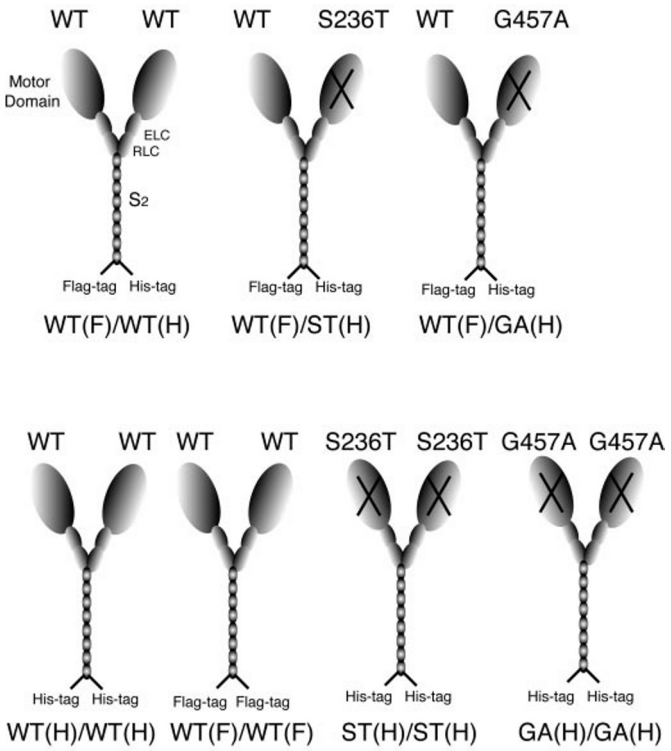

FIG. 1. **Schematic smooth muscle myosin constructs.** Asymmetric HMM, WT(F)/ST(H), and WT(F)/GA(H) have one wild-type head with C-terminal FLAG tag and one mutant (S236T or G457A) head with C-terminal His tag. WT(F)/WT(H) has two wild-type heads with a FLAG tag or His tag at each C terminus. Symmetric HMM, WT(F)/WT(F), has two wild-type heads with a FLAG tag at both C termini. WT(H)/WT(H), ST(H)/ST(H), and GA(H)/GA(H) are symmetric HMM with wild-type heads or mutant heads and C-terminal His tags.

heavy chains (*i.e.* WT(F)/WT(F)), respectively, were also expressed and purified using similar procedures, except that only one affinity chromatography was used for purification (*i.e.* Ni^{2+}-agarose for two His-tagged HMM and anti-FLAG M2 affinity agarose for two FLAG-tagged HMM). The purified HMMs were subjected to SDS-PAGE followed by Western blot analysis using anti-His antibodies and anti-FLAG antibodies as probes. As shown in Fig. 2*A*, WT(F)/WT(H) was recognized by both anti-His antibodies and anti-FLAG antibodies, respectively. On the other hand, WT(H)/WT(H) and WT(F)/WT(F) were recognized only by anti-His antibodies and anti-FLAG antibodies, respectively. Asymmetric HMMs, WT(F)/ST(H) and WT(F)/GA(H), were isolated by using similar procedures (Fig. 2). The Western blot analysis of the asymmetric HMMs revealed that these HMMs contain both His and FLAG tags in the molecule, indicating that these HMMs are the heterodimer composed of one wild-type heavy chain and one mutant heavy chain. Previously, we found that smooth muscle HMM mutants, S236T or G457A, bind to actin in the absence of ATP and dissociate from actin in the presence of ATP. Therefore, it is anticipated that the asymmetric HMM containing a mutant heavy chain can bind to actin in an ATP-dependent manner. To test this, we mixed the isolated HMMs with F-actin and ultracentrifuged the mixture in the presence and absence of ATP. Fig. 2*C* shows SDS-PAGE of the pellets and the supernatants of acto-HMM after centrifugation. Both asymmetric HMMs and WT HMM co-precipitated with actin in the absence of ATP, and the majority of HMMs was dissociated from actin in the presence of ATP and recovered in the supernatant. It should be noted that asymmetric HMM as well as WT HMM did not precipitate in the absence of actin (not shown).

ATPase Activity of the HMM Constructs—Table I shows the

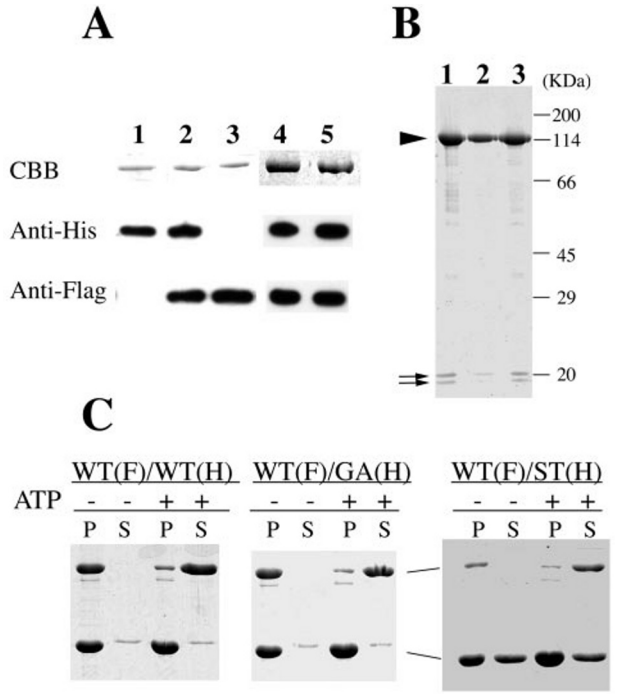

FIG. 2. **Purification of smooth muscle HMM.** Three asymmetric HMM, WT(F)/WT(H), WT(F)/ST(H), and WT(F)/GA(H), were purified by two sequential affinity columns, Ni^{2+}-agarose, and anti-FLAG M2 affinity gel chromatography. Symmetric HMM, WT(H)/WT(H), ST(H)/ST(H), and GA(H)/GA(H), were purified by Ni^{2+}-agarose chromatography. WT(F)/WT(F) was purified by anti-FLAG M2 affinity gel chromatography. *A*, Coomassie Brilliant Blue staining and Western blot of purified symmetric and asymmetric HMM with anti-His antibody or anti-FLAG antibody. *Lane 1*, WT(H)/WT(H); *lane 2*, WT(F)/WT(H); *lane 3*, WT(F)/WT(F); *lane 4*, WT(F)/ST(H); *lane 5*, WT(F)/GA(H). *B*, Coomassie Brilliant Blue staining of purified asymmetric HMM. The *arrow* and *arrowhead* indicate heavy chain and light chains, respectively. *Lane 1*, WT(F)/WT(H); *lane 2*, WT(F)/ST(H); *lane 3*, WT(F)/GA(H). *C*, actin-binding properties of asymmetric HMM. Purified asymmetric HMMs were mixed with 0.5 mg/ml actin in the absence (−) or presence (+) of 1 mM Mg^{2+}-ATP in a solution of 20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, and 1 mM DTT and subjected to centrifugation at 80,000 rpm at 4 °C for 10 min. The same portion of supernatant (*S*) and pellet (*P*) was analyzed by SDS-PAGE (7.5–20%) and stained by Coomassie Brilliant Blue. *Upper line*, HMM heavy chain; *lower line*, actin.

TABLE I
Ca^{2+}- and K^+(EDTA)-ATPase activity of asymmetric and symmetric HMMs

Ca^{2+}-ATPase activity was measured at 25 °C in 10 mM $CaCl_2$, 0.5 M KCl, 30 mM Tris-HCl pH 8.5. K^+(EDTA)-ATPase activity was measured at 25 °C in 10 mM EDTA, 0.5 M KCl, 30 mM Tris-HCl, pH 8.5.

	Ca^{2+}-ATPase	K^+(EDTA)-ATPase
	s^{-1}	
WT(F)/WT(H)	0.930	1.765
WT(F)/ST(H)	0.800	0.830
WT(F)/GA(H)	0.507	1.070
ST(H)/ST(H)	0.788	0.022
GA(H)/GA(H)	<0.003	<0.003

Ca^{2+}- and K^+(EDTA)-ATPase activity of the purified HMMs. ST(H)/ST(H) HMM showed nearly normal Ca^{2+}-ATPase activity but very low K^+(EDTA)-ATPase activity, which is consistent with our previous report (14, 15). On the other hand, GA(H)/GA(H) HMM had no detectable ATPase activity in either condition. This is also consistent with our previous result (15). K^+(EDTA)-ATPase activity of WT(F)/ST(H) HMM was about half of that of WT(F)/WT(H), whereas the Ca^{2+}-ATPase activity was similar to those of WT(F)/WT(H) HMM and ST(H)/ST(H) HMM. On the other hand, both Ca^{2+}-ATPase and K^+(EDTA)-ATPase activities of WT(F)/GA(H) HMM were ap-

TABLE II
Mg^{2+}-ATPase activity of asymmetric and symmetric HMMs

Assay conditions were 0.01–0.03 mg/ml HMM, 0.5 mg/ml BSA, 0.3 mM ATP, 24 mM KCl, 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 0 or 2 mg/ml actin. 0.2 mM $CaCl_2$, 15 μg/ml MLCK, and 10 μg/ml calmodulin were added to measure the activity in the phosphorylated state, whereas 1 mM EGTA was added in the unphosphorylated state.

	Unphosphorylated		Phosphorylated	
	0 mg/ml actin	2 mg/ml actin	0 mg/ml actin	2 mg/ml actin
WT(H)/WT(H)	0.014 ± 0.002	0.043 ± 0.011	0.042 ± 0.015	1.306 ± 0.056
WT(F)/WT(F)	0.013	0.033	0.034	0.935
WT(F)/WT(H)	0.013 ± 0.001	0.052 ± 0.013	0.044 ± 0.015	0.840 ± 0.134
ST(H)/ST(H)	0.025	0.050	0.078	0.069
WT(F)/ST(H)	0.025 ± 0.005	0.068 ± 0.026	0.048 ± 0.022	0.340 ± 0.017
GA(H)/GA(H)	0.002	0.004	0.002	0.004
WT(F)/GA(H)	0.012 ± 0.004	0.034 ± 0.002	0.030 ± 0.009	0.096 ± 0.018

proximately half of those of the wild-type HMM. These results suggest that the ATPase activities in these conditions are independent of the activity of the partner head.

To assess the phosphorylation-dependent regulation of the motor function of the constructs, we measured the actin-activated ATPase activities of HMMs. As expected, the actin-activated ATPase activities of all wild-type HMM (*i.e.* WT(H)/WT(H), WT(F)/WT(F), and WT(F)/WT(H)) were well regulated by phosphorylation (*i.e.* little actin-activated ATPase activity in the unphosphorylated state and high actin-activated ATPase activity in the phosphorylated state) (Table II). On the other hand, ST(H)/ST(H) HMM showed virtually no phosphorylation dependence on the actin-activated ATPase activity, although the basal ATPase activity was retained. GA(H)/GA(H) HMM showed hardly any ATPase activity in all conditions (Table II).

Fig. 3 shows the actin-activated ATPase activity of asymmetric HMMs as a function of actin concentration. The actin-activated ATPase activities of asymmetric HMMs were increased by phosphorylation; however, the activities were significantly lower than that of WT(F)/WT(H) HMM. The V_{max} of the actin-activated ATPase activities of phosphorylated WT(F)/ST(H) HMM and WT(F)/GA(H) HMM were 0.404 and 0.156 s^{-1}, respectively. These values are much lower than half the value of the phosphorylated WT(F)/WT(H) HMM (1.85 s^{-1}) (*i.e.* 0.93 s^{-1}) (Table III). This implies that the presence of an inactive head significantly attenuates the activity of the wild-type head. In other words, two functional heads are required for the full activation of smooth muscle myosin II. It has been pointed out that the steady-state values of the actin-activated ATPase activity may underestimate the extent of phosphorylation-dependent regulation of myosin, because a minor contamination of the unregulated myosin having a high ATPase activity would significantly increase the apparent ATPase activity of the dephosphorylated myosin (12). On the contrary, single turnover measurement could provide more accurate information on the regulation, even if the samples contain a minor fraction of unregulated components. Therefore, we performed a single turnover experiment of the actin-activated ATPase reaction of thiophosphorylated WT(F)/GA(H) HMM and WT(F)/WT(H) HMM by using Mant-2-dATP as a substrate (Fig. 4). HMM was first mixed with of Mant-2-dATP to form HMM-ADP-P complex, and then actin was added. The release of the product was monitored by measuring the decrease in the fluorescence intensity of Mant nucleotide. The decrease in fluorescence intensity could be best fitted with two exponentials for WT(F)/WT(H) HMM. The weighted single turnover rate for thiophosphorylated WT(F)/WT(H) HMM (0.359 s^{-1}) was very similar to the steady-state rate in the presence of 13.3 μM actin. For phosphorylated WT(F)/GA(H) HMM, the fluorescence decrease of Mant moiety could be fit in two exponentials. The weighted single turnover rate for thiophosphorylated WT(F)/GA(H) HMM (0.0737 s^{-1}) was much lower than that of thiophosphorylated WT(F)/WT(H) HMM (Table IV). It should be noted that GA(H)/GA(H) HMM showed virtually no hydrolysis.

In Vitro Actin-gliding Activity—To determine the mechanical activity of the asymmetric HMMs directly, we performed an actin surface-gliding assay. Consistent with previous reports (14, 15), no actin-gliding activity was found for thiophosphorylated homodimer G457A mutant, GA(H)/GA(H), and very low actin-gliding activity (<0.01 μm/s) was found for thiophosphorylated homodimer S236T mutant, ST(H)/ST(H) (not shown). As shown in Fig. 5, thiophosphorylated wild-type HMM, WT(F)/WT(H), moved actin filament smoothly at a velocity of 0.27 ± 0.03 μm/s at 25 °C. On the other hand, the actin-gliding velocities of thiophosphorylated asymmetric HMMs, both WT(F)/ST(H) HMM and WT(F)/GA(H) HMM, were only one-seventh to one-sixth and one-thirteenth of that of wild-type HMM, respectively. We also examined whether the presence of inactive HMM interferes with the movement of actin in the surface gliding assay. We mixed an equal amount of the thiophosphorylated WT(H)/WT(H) HMM and the thiophosphorylated GA(H)/GA(H) HMM and applied it onto the flow cell to perform the actin-gliding assay. As shown in Fig. 5, the actin-gliding velocity was not significantly decreased by the presence of the inactive mutant HMM. Therefore, the slow actin-gliding velocity of the phosphorylated WT(F)/GA(H) should be due to the negative influence of the inactive head to the wild-type head of HMM.

DISCUSSION

Our results clearly demonstrate that the two functionally active heads are required for full activation of smooth muscle myosin motor activity in the phosphorylated state. It was shown previously that the mutation of Ser-236 in the Switch I loop to Thr abolishes the actin-activated ATPase activity of phosphorylated smooth muscle myosin II although the S236T mutant has normal ATP hydrolysis, thus forming myosin-ADP-P intermediate (14). It was also shown that the mutation of Gly-457 in the Switch II loop to Ala abolishes ATP hydrolysis of myosin II, thus inactivating the motor activity completely, although the mutation does not hamper the ATP binding and thus the ATP-induced dissociation of myosin II from actin (15). The present results for the mutant homodimers are consistent with these earlier findings. The most important finding in the present study is that the wild-type head of the HMM heterodimer having a mutant head exhibited significantly lower actin-activated ATPase activity than the wild-type heads in the homodimer. The reduced activity of the wild-type head was more dramatic in HMM having a G457A mutant head. We think that the decrease in the actin-activated ATPase activity of the phosphorylated wild-type head in heterodimer HMM is due to the disruption of the proper interaction between the heads. In other words, the phosphorylated wild-type HMM heads interact with each other, which is critical for full activa-

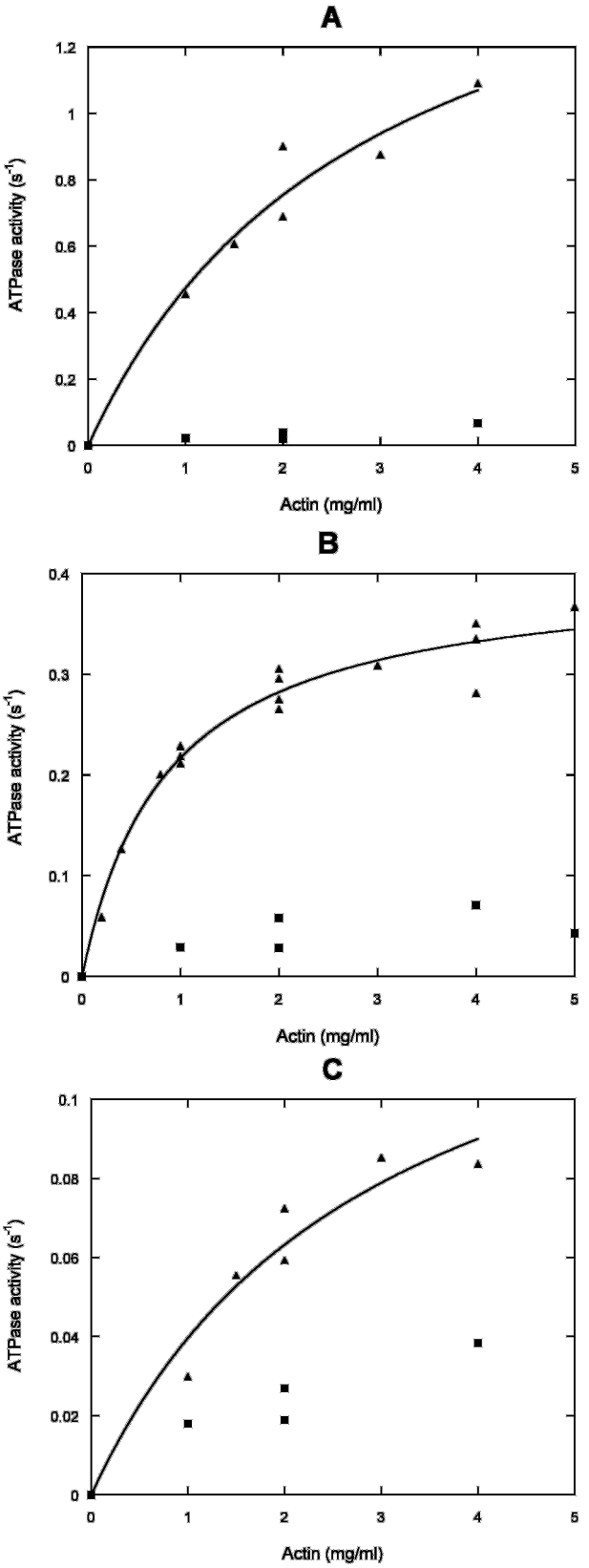

FIG. 3. **Actin-activated ATPase activity of asymmetric HMM.** *A*, WT(F)/WT(H); *B*, WT(F)/ST(H); *C*, WT(F)/GA(H). Assay conditions were 0.01–0.03 mg/ml HMM, 0.5 mg/ml bovine serum albumin, 0.3 mM ATP, 24 mM KCl, 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and various concentrations of actin. 0.2 mM $CaCl_2$, 15 μg/ml MLCK, and 10 μg/ml calmodulin were added to measure the activity in the phosphorylated state, whereas 1 mM EGTA was added in the unphosphorylated one. For each actin concentration, the time course of the phosphate liberation up to 60 min was measured. The phosphorylation of RLC in the myosin constructs by MLCK in the same condition was saturated within 15 s (not shown). *Squares* represent unphosphorylated state, whereas *triangles* represent the phosphorylated state. *Curves* are the least squares fits of the data points based upon the equation, $V = (V_{max} * [actin])/(K_{actin} + [actin])$. K_{actin} and V_{max} are listed in Table III.

TABLE III
V_{max} and K_{actin} of actin-activated ATPase activity of phosphorylated asymmetric HMM

Assay conditions were as described in the legend to Fig. 3. Basal activity was deducted. Curves are the least squares fits of the data points based upon the equation, $V = (V_{max} * [actin])/(K_{actin} + [actin])$. Similar results were obtained for two independent preparations of WT(F)/WT(H) and WT(F)/GA(H) and three independent preparations of WT(F)/ST(H).

	V_{max} (s^{-1})	K_{actin} (mg/ml)
WT(F)/WT(H)	1.85	2.85
WT(F)/ST(H)	0.404	0.86
WT(F)/GA(H)	0.156	2.93

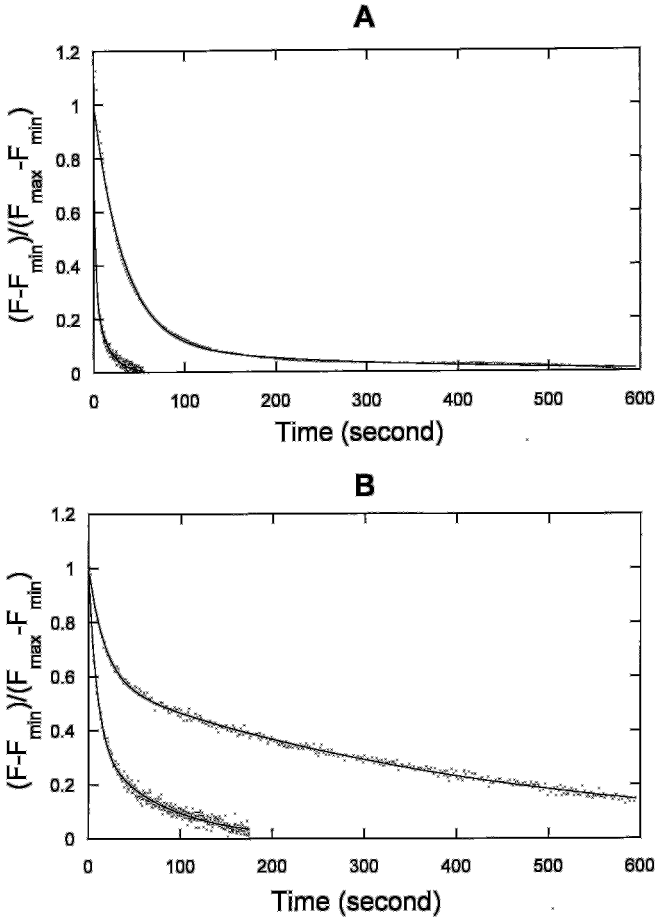

FIG. 4. **Single turnover ATPase assay of thiophosphorylated asymmetric HMM.** *A*, WT(F)/WT(H); *B*, WT(F)/GA(H). The release of Mant nucleotide from the myosin-ADP-P_i complex was measured in the presence of 0 μM (*upper line*) or 13.3 μM actin (*lower line*) as described under "Experimental Procedures." The rate constants and fractional amplitudes of each fitting are listed in Table IV.

tion of the actin-activated ATPase activity, whereas the inactive mutant head cannot properly interact with the neighboring wild-type head. The results indicate that interhead interaction is required for the full activation of the phosphorylated smooth muscle myosin. It was shown previously that the motor activity of smooth muscle myosin composed of a complete head and a regulatory light chain binding domain shows an actin-activated ATPase activity significantly lower than that of

TABLE IV
Single turnover ATPase assay of thiophosphorylated WT(F)/WT(H) and WT(F)/GA(H)

Single turnover rates were measured, and data were fit as described under "Experimental Procedures." Data were fit to a double exponential model ($A_1 * e^{-k1*t} + A_2 * e^{-k2*t}$), because a single exponential model consistently missed a fast phase.

	WT(F)/WT(H)		WT(F)/GA(H)	
	0 μM actin	13.3 μM actin	0 μM actin	13.3 μM actin
A_1	0.897	0.537	0.415	0.652
k_1 (s^{-1})	0.0304	0.516	0.0552	0.106
A_2	0.103	0.293	0.585	0.348
k_2 (s^{-1})	0.0036	0.0707	0.00230	0.0131
k_{wt} (s^{-1})[a]	0.0276	0.359	0.0245	0.0737

[a] k_{wt}, weighted single turnover rate, is calculated by the equation, $k_{wt} = (A_1*k_1 + A_2*k_2)/(A_1 + A_2)$.

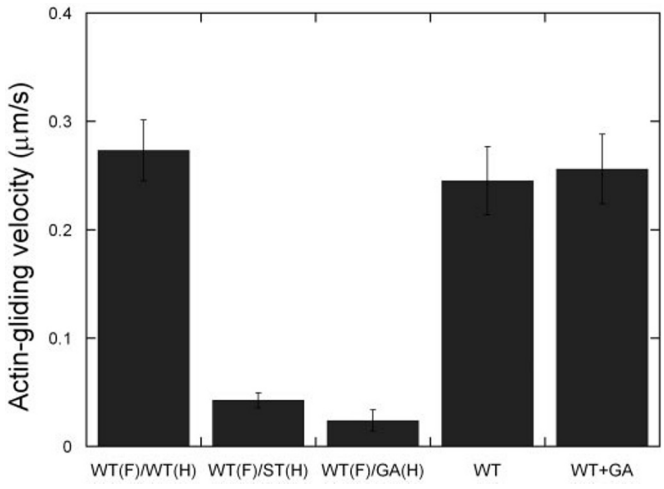

FIG. 5. **Actin-gliding velocity of thiophosphorylated asymmetric and symmetric HMM.** Actin movement was observed in 30 mM KCl, 5 mM $MgCl_2$, 25 mM imidazole, 1 mM EGTA, 1% 2-mercaptoethanol, 0.5% methylcellulose, 4.5 mg/ml glucose, 216 μg/ml glucose oxidase, 36 μg/ml catalase, 2 mM ATP, at 25 °C and pH 7.5. All values are mean velocities ± S.D. WT(F)/WT(H), WT(F)/ST(H), and WT(F)/GA(H) represent asymmetric HMM. *WT* represents wild-type HMM with His tag (*i.e.* WT(H)/WT(H)), whereas *WT + GA* represents a mixture of the same concentration of WT(H)/WT(H) and GA(H)/GA(H). All samples are thiophosphorylated by MLCK as described previously (8).

HMM having complete two heads, although the ATPase activity is enhanced by RLC phosphorylation (11, 13). The present results are consistent with these earlier reports and indicates that the interaction between the two heads at the motor domain is important to obtain the full activation by phosphorylation.

Quite interestingly, the activation of the ATPase activity of the wild-type head was hampered more prominently in the presence of the G457A mutant head than the S236T mutant head. The former mutation inhibits ATP hydrolysis, thus forming myosin-ATP as a stable intermediate, whereas the latter mutant can form myosin-ADP-P as a stable intermediate; therefore, it is suggested that the conformational change upon ATP hydrolysis is critical to induce the cooperativity between the heads that leads to phosphorylation-induced activation.

It is of interest that the decrease in the ATPase activity of the wild-type head in the heterodimer HMM was only observed for the actin-activated ATPase activity in the phosphorylated state, and the basal ATPase activity as well as Ca^{2+}- and K^+(EDTA)-ATPase activity were not influenced by the presence of the inactive head. This suggests that the cooperative activation of HMM involves the binding of the heads to actin. There are two possibilities to account for the nature of the cooperativity. One is that the two heads directly interact with each other, increasing the ATPase turnover rate. The other is that the binding of the one head to actin facilitates the binding of the other head to actin to enhance the product release, thus facilitating the cycle. It has been shown that dephosphorylated smooth muscle myosin heads interact with each other at the converter domain of the one head with the motor core domain of the other head (22). Similar interaction between the heads may be operating in the heterodimer HMM in the present study. However, further studies are required to clarify the mechanism underlying the requirement of the two active heads for full activity of smooth muscle myosin II.

The single turnover data of the thiophosphorylated WT HMM was fitted by the two rate constants. There is a possibility that the slow product release is due to some dead myosin present in the preparation. However, we think this is less likely, because the fraction of the slow component does not markedly vary from preparation to preparation. The slow product release component was also reported by Ellison *et al.* (23). Previously, it was shown that the product release of skeletal myosin subfragment 1 is fitted with two rate constants at saturating actin concentration, and it was explained that the slower rate constant is due to the slow ATP hydrolysis step of the actin-bound myosin head (24). The slower rate constant in Fig. 4 may be explained in part due to the similar process. However, it is anticipated that the majority of the myosin heads are dissociated from actin in the experimental conditions used, and it is less likely that the slower rate constant is entirely due to the hydrolysis step of the actin-bound myosin heads. Alternatively, the slower rate constant may be due to the product release from myosin-ADP-P without binding to actin, whereas the faster rate constant is via the actin binding process. Since GA(H)/GA(H) HMM showed virtually no hydrolysis, we think that both the fast and slow components of the asymmetric HMM represent the product release from the wild type head.

The actin-gliding velocity of HMM having the mutant head was much lower than the wild-type HMM, and the difference was much more pronounced than that for the actin-activated ATPase activity. This is not due to the presence of the inactive heads on the coverslip during the actin-gliding assay, because the presence of GA(H)/GA(H) on the glass surface did not decrease the velocity of actin filaments by WT(H)/WT(H).

In order to move actin filaments, each head of myosin must cycle repetitively between attached and detached phases. In the attached phase, of duration T_{on}, the head undergoes the "working" stroke, and in the detached phase, of duration T_{off}, it undergoes the "recovery" stroke, which returns the head to its initial conformation (25). By recovering during the detached phase, the myosin motor avoids stepping back and so progresses through the working distance during each cycle. The actin-gliding velocity (ν) is determined by the working distance (δ) and attached duration (T_{on}), $\nu = \delta/T_{on}$. Therefore, the high actin-gliding velocity of double-headed HMM (WT(F)/WT(H)) could be due to the large working distance and/or short attached duration. Tyska *et al.* (26) measured the unitary displacement and force produced by a single molecule of the double-headed and single-headed smooth muscle myosins randomly oriented on the coverslip. Single-headed myosin produces approximately half of the displacement and force of double-headed myosin during a unitary interaction with actin, and it was proposed that muscle myosins require both heads to generate maximal force and motion, although this may be due to the random orientation of the heads (27). The very low actin-gliding velocity of WT(F)/GA(H) may partially be explained by the decrease in the working distance in addition to the slow turnover rate of WT(F)/GA(H).

The turnover rate of ATP hydrolysis by myosin is determined by the combination of the duration of attached phase and the detached phase (turnover rate = $1/T_{total} = 1/(T_{on} + T_{off})$). During the cross-bridge cycling, smooth muscle myosin heads spend a majority of the time in the detached state ($T_{off} \gg T_{on}$). Therefore, the turnover rate of smooth muscle myosin is largely determined by the detached phase. If the attachment of the first head on actin facilitates the attachment of the second head to actin, the overall duration for each turnover would be decreased, thus increasing the turnover rate for two-headed myosin. It is likely that this kind of cooperativity only exists between two heads of phosphorylated smooth muscle HMM. Direct chemical cross-linking and three-dimensional image reconstruction of smooth muscle HMM have revealed that the orientation and geometry of two heads is dependent on the phosphorylation state (22, 28). Therefore, it is plausible that the significant decrease in the turnover rate of the heterodimer HMM having an inactive head is due to the lack of the cooperative binding to actin.

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