Myosin VI is expressed in a variety of cell types and is thought to play a role in membrane trafficking and endocytosis, yet its motor function and regulation are not understood. The present study clarified mammalian myosin VI motor function and regulation at a molecular level. Myosin VI ATPase activity was highly activated by actin with $K_{\text{act}}$ of 9 $\mu$M. A predominant amount of myosin VI bound to actin in the presence of ATP unlike conventional myosins. $K_{\text{ATP}}$ was much higher than those of other known myosins, suggesting that myosin VI has a weak affinity or slow binding for ATP. On the other hand, ADP markedly inhibited the actin-activated ATPase activity, suggesting a high affinity for ADP. These results suggested that myosin VI is predominantly in a strong actin binding state during the ATPase cycle. p21-activated kinase 3 phosphorylated myosin VI, and the site was identified as Thr606. The phosphorylation of myosin VI significantly facilitated the actin-translocating activity of myosin VI. On the other hand, Ca$^{2+}$ diminished the actin-translocating activity of myosin VI although the actin-activated ATPase activity was not affected by Ca$^{2+}$. Calmodulin was not dissociated from the heavy chain at high Ca$^{2+}$, suggesting that a conformational change of calmodulin upon Ca$^{2+}$ binding, but not its physical dissociation, determines the inhibition of the motility activity. The present results revealed the dual regulation of myosin VI by phosphorylation and Ca$^{2+}$ binding to calmodulin light chain.

Myosin, a motor protein that translocates actin filaments upon hydrolysis of ATP, constitutes a superfamily with 18 classes based upon phylogenetic sequence comparisons of the motor domains (1–4). Class VI myosins were first identified in Drosophila melanogaster (5), and subsequently found in mammals (6–8). While myosin VI is found in various tissues, its physiological significance may be felt most prominently in auditory function, where it is found that the mutation of the myosin VI gene results in auditory malformation (7, 8). Actually, myosin VI is found in the neuroepithelium of the cochlea of the inner ear in both the inner and outer hair cells. In these cells, myosin VI is concentrated at actin-rich stereocilia and may play a role in the rigidity of anchoring of the stereocilia (7, 8); therefore, it has been suggested that myosin VI plays a role in the mechanical function of stereocilia (7, 8). In Drosophila, myosin VI is associated with particles that move in a cell cycle-dependent manner (9, 10). It has also been suggested that myosin VI plays a role in membrane trafficking and endocytosis (11).

The structure of myosin VI has been predicted based upon its amino acid sequence to be composed of a head domain, a coiled-coil domain, and a globular tail domain. The head domain is itself divided into a globular motor domain and a neck domain containing a light chain binding region. The sequence at the neck region contains a single IQ motif that is implicated as a calmodulin or myosin light chain binding consensus motif as found in a variety of calmodulin-binding proteins and myosins (1–4). The coiled-coil domain is present at the C-terminal side of the neck region, so it is predicted that myosin VI is a two-headed myosin. Finally, the globular tail domain is hypothesized to be a targeting domain that determines the cellular binding counterpart.

There are two unique inserts in the head domain, one at the surface in the upper 50-kDa domain and the other at the junction between the converter domain and the IQ domain. Until quite recently, all myosin motors were characterized as moving toward the barbed end of actin filaments. Since unconventional myosins play a role in translocating cellular organelles along actin cables, a myosin having opposite moving directionality would be expected to have unique cellular functions. Of the 18 different classes of myosins, it was found that myosin VI moves toward the minus end of F-actin filaments (12). While the mechanism underlying the reverse movement of myosin VI is unclear, the unique large insertion in the myosin VI head domain between the motor domain and the light chain binding domain (lever arm) has led to the postulation (12) that this alters the angle of the lever arm switch movement, thus changing the direction of motility.

The importance of light chains in the regulation of myosin motor function is seen in both vertebrate smooth muscle/non-muscle myosin and invertebrate myosin, in which the phosphorylation of the regulatory light chain (13–15) and Ca$^{2+}$ binding to the essential light chain (16), respectively, trigger the activation of motor activity.

The role of the IQ motif and bound calmodulin serving as a regulatory component of unconventional myosins was first studied for mammalian myosins I. For both brush border myosin I (17, 18) and myosin Ib (19–21), high Ca$^{2+}$ inhibits motor activity due to its binding to the calmodulin light chain. Since 1 mol of bound calmodulin dissociates from myosin I at high Ca$^{2+}$, it was originally thought that this dissociation of calmodulin was responsible for the inhibition of myosin I motor activity (17, 18). However, since virtually no calmodulin dissociation is observed at pCa 6, where the motility activity is completely abolished, this view has been questioned (20, 21). Quite recently, a similar finding was made for myosin V (22). While calmodulin can be dissociated from its heavy chain at high Ca$^{2+}$, the motility activity of myosin V is abolished at pCa 6, where no calmodulin dissociation takes place, suggesting that a Ca$^{2+}$ binding-induced conformational change of the bound cal-
modulin, but not a physical dissociation, is critical for regulation. However, most calmodulin-binding myosins contain multiple calmodulin molecules at their neck domains, and it has been shown that neck-deleted myosin V containing a single bound calmodulin is unregulated by Ca^2+ (22, 23). Therefore, a remaining question is whether or not the motor activity of myosin VI, which contains a single calmodulin at its neck domain, is regulated by Ca^2+.

Myosin VI contains a potential phosphorylation site in a loop near the tip of the head that is in a homologous position to the amoeba myosin I phosphorylation site (24). Since the phosphorylation of the heavy chain of amoeba myosin I at this site is necessary for the activation of both actin-activated ATPase activity and motility activity (25), a hypothesis is raised that myosin VI motor function might be regulated by the phosphorylation at this position by protein kinases, although no supporting data are available to date.

The aim of the present study is to clarify the motor function and regulation of myosin VI. To achieve the goal, we expressed myosin VI HMM in SF9 cells, purified and examined for motor function. The obtained results reveal a dual regulation of myosin VI motor function by Ca^2+ binding and p21-activated kinase 3-catalyzed phosphorylation.

**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 (26). Recombinant calmodulin from *Xenopus* oocyte (27) was expressed in *Escherichia coli* as described (28).

**Generation of the Expression Vectors for Myosin VI Constructs**—Mouse cDNA clones containing ~150 to 2565 (B10) and 1460–3708 (B2) in pBluescript were kindly provided by Dr. K. Avraham (Tel Aviv University). B10 clone was digested by *Kpn*I/*Pml*I, and the cDNA fragment of myosin VI (residues 1679–3309) obtained from the B2 clone by *Kpn*I/*M1 digestion was inserted into the B10 clone (B2/B10). A unique *Nhe*I site was created at the 5′ side of the initiation codon, and then the myosin VI cDNA by *Nhe*I/*Kpn*I digestion was inserted into *pBluebac 4* (Invitrogen, Carlsbad, CA) baculovirus transfer vector at the polylinker region. A hexahistidine tag sequence with a stop codon was inserted next to the coding sequence of actin expressing baculovirus transfer vector at the N-terminal of the myosin VI HMM coding region. The obtained construct (M6HMM), containing the entire coding region was in-frame ligated into pBluescript vector. This construct (M6HMM) was used as the template to express PAK3 kinase domain (PAK KD) by using a spliced leader from *Bomastom* (Bomastomis japonicus) vector. A fragment containing the entire coding region was in-frame ligated into *pFastBac HTb* (Life Technologies, Inc.) baculovirus transfer vector. To produce a constitutively active form, a *Bam*HI site was created at codon 210, and the nucleotides encoding the N-terminal 210 amino acid residues were excised by *Bam*HI digestion. The produced construct was used to express PAK3 kinase domain (PAK KD). The plasmids were transformed into DH10BAC *E. coli*. Recombinant bacmids (recombimab vector DNA) were isolated and used to transfect SF9 insect cells.

**Preparation of Recombinant Myosin VI**—To express recombinant myosin VI, 200 ml of SF9 cells (about 1 × 10^8) were infected. The cells were cultured at 28°C in 175-mm flasks and harvested after 3 days. Cells were lysed with sonication in 30 ml of lysis buffer (0.5 M NaCl, 30 mM Tris-HCl, pH 7.5, 2 mM MgCl_2, 1 mM EGTA, 2 mM β-metcaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml trypsin inhibitor, 10 mM β-metcaptoethanol). After centrifugation at 140,000 × g for 15 min, the supernatant was mixed with 0.5 ml of Ni⁺⁺-nitritotriacetic acid-agarose and incubated for 30 min on a rotating wheel for 4°C. The resin was then washed with 500 × g for 3 min and then suspended in buffer E (0.5 M NaCl, 30 mM Tris-HCl (pH 7.5), 10 mM β-metcaptoethanol, 10 μM leupeptin). This resin suspension was loaded on a column (1 × 10 cm) and was washed with a 30-fold volume of buffer C. PAK3KD was eluted with buffer F (0.5 M NaCl, 200 mM imidazole-HCl (pH 7.5), 10 mM β-metcaptoethanol, 10 μM leupeptin).

**Generation of Antibody**—A peptide (12-mer) containing phospho-Thr^406^ of N-terminal CS was chemically synthesized as antigen and bound to the carrier protein, keyhole limpet hemocyanin at the N-terminal cyanine residue. Antibodies were prepared by injecting two rabbits with keyhole limpet hemocyanin-coupled peptide. Antibodies against phospho-Thr^406^ were affinity-purified from the obtained serum by affinity chromatography with the peptide-conjugated resin. The purified antibodies were subjected to absorption on unphosphorylated p21-activated kinase 3 (PAK3).

**Gel Electrophoresis and ATPase Assay**—SDS-polyacrylamide gel electrophoresis was carried out on a 7.5–20% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli (31). Molecular mass markers used were smooth muscle myosin heavy chain (204 kDa, β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and ovalbumin (45 kDa), human albumin (66 kDa), and carbonic anhydrase (29 kDa).

**Phosphorylation of Myosin VI by PAK3**—To phosphorylate myosin VI, 200 ml of SF9 cells (about 1 × 10^8) were infected. The cells were cultured at 28°C in 175-mm flasks and harvested after 3 days. Cells were lysed with sonication in 30 ml of lysis buffer (0.5 M NaCl, 30 mM Tris-HCl, pH 7.5, 2 mM MgCl_2, 1 mM EGTA, 2 mM β-metcaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, 0.1 mg/ml trypsin inhibitor, 10% glycerol, and 1 mM ATP). After centrifugation at 140,000 × g for 15 min, the supernatant was incubated with 50 μM microcystin, 1 mM dithiothreitol, and 0.2 μM microcin for 30 min at 25°C (35). The purified myosin VI heavy chain and calmodulin was determined by densitometry as described previously (20). The steady-state ATPase activity was determined by measuring liberated P_i at 25°C as described previously (32). The ATPase activity was also measured in the presence of 20 units/ml pyruvate kinase, 3 mM phosphoenolpyruvate as a ATP regeneration system. The liberated pyruvate was determined as described (33).

**In Vitro Motility Assay**—The in vitro motility assay was performed as described previously (34). Myosin VI was attached to a coverslip. Actin filament velocity was calculated from the movement distance and the elapsed time in successive snapshots. A Student’s t test was used for statistical comparison of mean values. A value of p < 0.01 was considered to be significant.

**Phosphorylation of Myosin VI by PKA3**—Prior to the phosphorylation reactions, PAK3KD was incubated in 75 mM NaCl, 30 mM imidazole-HCl (pH 7.5), 4 mM MgCl_2, 1 mM ATP, 2 mM dithiothreitol, and 0.2 μM microcin for 30 min at 25°C. The phosphorylation reaction was then carried out in a buffer containing 50 mM NaCl, 20 mM imidazole-HCl (pH 7.5), 2 mM MgCl_2, 0.1 μM microcin, 1 mM dithiothreitol, 0.5 mM f-ATP (300 μM) in the presence of 4 μg of myosin VI. The reaction was stopped by adding 5% trichloroacetic acid and analyzed by SDS-PAGE and autoradiography. To quantify phosphate incorporation, the myosin VI heavy chain bands of the SDS-PAGE gels were excised, and the radioactivity was quantitated by a scintillation counter.

**Western Blotting**—Western blotting was carried out as described previously (36).
Actin Co-sedimentation Assay—The binding of calmodulin to M6HMM heavy chain was determined by actin co-sedimentation assay. M6HMM was incubated in buffer containing 25 mM imidazole-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.025 mg/ml F-actin, and various concentrations of CaCl₂ at 25 °C for 15 min. The sample was ultracentrifuged at 100,000 × g for 30 min, and the pellets were analyzed by SDS-polyacrylamide gel electrophoresis. The amounts of the co-sedimented M6HMM heavy chain and calmodulin were determined by densitometry as described previously (20).

To determine the binding of myosin VI with actin in the presence of ATP, M6HMM (0.1 mg/ml) was incubated in buffer containing 20 mM imidazole, pH 7.5, 50 mM KCl, 2 mM MgCl₂, 2 mM ATP, and various concentrations of actin in the presence of ATP regeneration system (20 units/ml pyruvate kinase and 3 mM phosphoenolpyruvate) at 25 °C for 30 min. The samples were ultracentrifuged at 100,000 × g for 30 min, and the pellets were analyzed by SDS-polyacrylamide gel electrophoresis. The amount of the co-sedimented heavy chain was determined by densitometry as described previously (20).

RESULTS

Expression and Purification of Mammalian Myosin VI—Mouse myosin VI construct was produced and expressed in Sf9 insect cells. The construct (M6HMM) contains the entire coiled-coil domain and the complete head domain along with a C-terminal hexahistidine tag to aid in purification (Fig. 1). Histidine tagging at the C-terminal end of the molecule has been performed on conventional (37) as well as unconventional myosins, and no influence on motor function has been observed. The cells were co-infected with an appropriate ratio of myosin VI-expressing virus and calmodulin-expressing virus. It should be noted that functional myosin VI was only obtained with co-infection of calmodulin virus, in contrast to myosin V in which functional protein can be obtained without calmodulin co-infection (23). The purification process was basically two steps (i.e. F-actin co-precipitation followed by ATP-induced dissociation from F-actin and Ni²⁺-agarose affinity chromatography using the hexahistidine tag (see “Experimental Procedures”). The former step selects the functionally active molecules, and the second step eliminates the endogenous Sf9 cell myosin and F-actin.

Phosphorylation of Myosin VI by PAK3—Based upon the sequence alignment of myosin VI with other myosins, it was noticed that mammalian myosin VI contains a potential phosphorylation site (Thr⁴⁰⁶) in a loop near the tip of the head. It has been shown that the phosphorylation at a homologous position of the amoeba myosin I catalyzed by a PAK family kinase is required for its motor activity (i.e. its actin-activated ATPase activity and motility activity) (24, 25). We therefore investigated whether myosin VI is phosphorylated at Thr⁴⁰⁶. Fig. 3 shows the phosphorylation of myosin VI by mammalian PAK3KD. Myosin VI heavy chain was stoichiometrically phosphorylated (Fig. 3A). To identify the site phosphorylated by PAK3KD, the phosphorylated myosin VI was subjected to Western blot analysis using anti-phosphothreonine 406-specific antibodies (Fig. 3B). The antibodies recognized the myosin VI heavy chain only after PAK3KD-induced phosphorylation but not before the phosphorylation. The signal strength of the Western blot increased over time with PAK3KD-induced phosphorylation, and the time course of the signal increase was similar to that of the extent of ³²P incorporation. Furthermore, the phosphorylation (incorporation of ³²P) by PAK3KD was markedly attenuated when the T406A mutant was used as a

— M. Ikebe, unpublished observation.
mammalian myosin V and myosin I as a function of Ca²⁺VI (Figs. 4 and 5; Table I). Furthermore, Ca²⁺ and myosin I (17–19) were also found to be activated by actin. The actin concentration dependence of M6HMM ATPase activity. The ATPase activity was not affected by the presence of ADP (Fig. 6). Ca²⁺ concentrations and then ultracentrifuged to determine bound calmodulin. F-actin co-precipitated myosin VI with bound calmodulin was analyzed by SDS-PAGE, and the calmodulin band was quantitated by densitometry and normalized to myosin VI heavy chain. As a control, free calmodulin was centrifuged with F-actin, but no calmodulin co-precipitation was detected. The myosin VI construct was also tested for phosphorylation by PK3KD at Thr⁴⁰⁶.

### Actin-activated ATPase Activity

Fig. 4 shows the actin dependence of M6HMM ATPase activity. The ATPase activity was markedly activated by actin. The actin concentration dependence of the activity showed a single saturation curve, and a Kₐ₅ₐ₉ of 9 μM was obtained. The value is significantly lower than those of nonprocessive conventional myosins. The actin-activated ATPase activity of M6HMM was also measured as a function of ATP concentration. As shown in Fig. 5, an extremely high concentration was required for the saturation of the steady state ATPase activity, and a Kₐ₅ₐ₉ value of 150 μM was obtained. The effect of the phosphorylation at Thr⁴⁰⁶ on the actin-activated ATPase activity was also studied (Figs. 4 and 5). M6HMM was phosphorylated by PK3KD to stoichiometric level prior to the ATPase assay. The ATPase activity was not significantly affected by the phosphorylation, and similar values of Vₐ₅₉, Kₐ₅₉, and Kₐ₅₉ were obtained for the phosphorylated and dephosphorylated M6HMM (Table I).

The actin-activated ATPase activity was measured as a function of free Ca²⁺ (Fig. 6). Ca²⁺ had no effect on the actin-activated ATPase activity in contrast to other calmodulin binding unconventional myosins so far reported such as myosin V and myosin I (17–23). Furthermore, Ca²⁺ changed neither Kₐ₅₉ nor Kₐ₅₉ of the actin-activated ATPase activity of myosin VI (Figs. 4 and 5; Table I).

Since the change in the actin-activated ATPase activity of mammalian myosin V and myosin I as a function of Ca²⁺ has been attributed to the dissociation of bound calmodulin (17, 18, 23), we examined the dissociation of calmodulin from myosin VI. The myosin VI construct was mixed with F-actin in various free Ca²⁺ concentrations and then ultracentrifuged to determine bound calmodulin. F-actin co-precipitated myosin VI with bound calmodulin was analyzed by SDS-PAGE, and the calmodulin band was quantitated by densitometry and normalized to myosin VI heavy chain. As a control, free calmodulin was centrifuged with F-actin, but no calmodulin co-precipitation was detected. The myosin VI construct was also tested for phosphorylation, but no myosin VI was precipitated in the absence of F-actin. As shown in Fig. 7, the amount of calmodulin bound to myosin VI was unchanged with Ca²⁺ increase. Consistently, the addition of exogenous calmodulin did not change the actin-activated ATPase activity (Fig. 6). These results suggest that calmodulin does not dissociate from the myosin VI heavy chain when its binding of Ca²⁺ induces a conformational change.

### Inhibition of Actomyosin VI ATPase Activity by ADP

It has been shown that low concentration of ADP significantly inhibits the actin-activated ATPase activity of myosin V, and this is
TABLE I
Effect of Ca$^{2+}$ and Thr$^{406}$ phosphorylation on the kinetic parameters of actomyosin VI ATPase activity

The actin-activated ATPase activity was measured as described in the legends to Figs. 4 and 5. The kinetic parameters were calculated from the data according to the equation $v = V_m [actin]/(K_{ATP} + [ATP])$, respectively.

| M6HMM | $K_{ATP}$ | $V_{max}$ | $K_{actin}$ | $V_{max}$ |
|-------|-----------|-----------|-------------|-----------|
|       | $\mu M$   | head$^{-1}$ s$^{-1}$ | $\mu M$ | head$^{-1}$ s$^{-1}$ |
| EGTA  |           |           |             |           |
| Dephosphorylated | 150 | 2.6 | 10 | 3.1 |
| Phosphorylated | 150 | 2.3 | 9.3 | 2.7 |
| pCa4  |           |           |             |           |
| Dephosphorylated | 160 | 2.1 | 8.7 | 2.0 |
| Phosphorylated | 150 | 1.9 | 7.4 | 1.8 |

Fig. 6. Actin-activated ATPase activity of M6HMM as a function of free Ca$^{2+}$. The actin-activated ATPase activity of M6HMM was measured in the presence of 13 $\mu M$ actin under the same conditions as described in the legend to Fig. 4, in the presence (●) and absence (●) of 12 $\mu M$ exogenous calmodulin.

Fig. 7. Dissociation of bound calmodulin from M6HMM. Purified M6HMM was mixed with F-actin in a buffer containing 25 mM KCl, 25 mM imidazole-HCl (pH 7.5), 5 mM MgCl$_2$, 1 mM EGTA, in the presence of various concentrations of Ca$^{2+}$, and then co-precipitated with F-actin by ultracentrifugation. F-actin co-precipitated M6HMM with bound calmodulin was resolved by SDS-PAGE. The molar ratio of bound calmodulin to M6HMM heavy chain was determined by densitometry analysis.

related to the strong binding of ADP to myosin V and the myosin V-ADP as a stable steady state intermediate (38, 39). Fig. 8 shows the time course of the actin-activated ATPase reaction of M6HMM in the presence and absence of ATP regeneration system. In the absence of the ATP regeneration system, the rate of Pi release was markedly inhibited. Based upon the curve fit with the equation, $v = V_m [ATP]/(K_{ATP} + [ATP])$ and the equation $v = V_m [actin]/(K_{actin} + [actin])$ and the equation $v = V_m [ATP]/(K_{ATP} + [ATP])$, respectively.

Fig. 8. Time course of the actin-activated ATPase activity of M6HMM with and without the ATP-regenerating system. ATPase activity was measured in the presence (●) and absence (●) of 20 units/ml pyruvate kinase and 3 mM phosphoenolpyruvate. Other assay conditions are as described in the legend to Fig. 4.

[ADP]/(K$_{ADP}$ + [ADP]) and $K_{ATP} = 150$ $\mu M$, $V_m = 3$ s$^{-1}$, and a $K_{ADP}$ of 3 $\mu M$ was obtained. The result indicates that M6HMM has a strong affinity for ADP.

Binding of Myosin VI and F-actin in the Presence of ATP—Binding of M6HMM to F-actin was directly determined by an F-actin co-precipitation assay. Myosin VI was mixed with F-actin in the presence of Mg$^{2+}$-ATP, and the amounts of the bound myosin VI and the dissociated myosin VI were determined after centrifugation (see "Experimental Procedures"). A majority of myosin VI was co-precipitated with F-actin in the presence of ATP (Fig. 9). The dissociation constant was estimated from the actin concentration dependence of the binding to be $K_d$ of 1.9 $\mu M$. It should be noted that M6HMM was readily dissociated from actin in the presence of Mg$^{2+}$-ATP at high ionic strength (0.5 M KCl). The result suggests that the stable steady state intermediate of the myosin VI ATPase cycle is the strong actin-binding form.

Effect of Ca$^{2+}$ on the Motility Activity of Myosin VI—To evaluate the motor activity of myosin VI more directly, the purified myosin VI was subjected to in vitro motility assay. Fig. 10 shows the motility activity of M6HMM at various calcium ion concentrations. The motility activity was completely inhibited at Ca$^{2+}$ concentrations higher than pCa 6. The inhibition of the motility was not reversed by the addition of exogenous calmodulin (up to 12 $\mu M$). The recovery of motility inhibited at high Ca$^{2+}$ was only achieved by reducing Ca$^{2+}$ concentration. The results are consistent with the fact that high Ca$^{2+}$ does not induce the dissociation of calmodulin from myosin VI heavy chain (Fig. 7) and suggest that the binding of Ca$^{2+}$ to the myosin VI-bound calmodulin light chain triggers the inhibition of the motility activity. While the reason for the apparent uncoupling between the ATPase activity and the motility activity at high Ca$^{2+}$ is unclear, it would be plausible that Ca$^{2+}$ might alter the affinity of myosin VI for actin. To address this possibility, the effect of Ca$^{2+}$ on actin-activated ATPase activity was measured as a function of actin concentration, but no significant effect of Ca$^{2+}$ was observed on $K_{actin}$ of the actin-activated ATPase activity of M6HMM (Fig. 4).

Effect of Thr$^{406}$ Phosphorylation on the Motility Activity of Myosin VI—The effect of Thr$^{406}$ phosphorylation on the motility activity of myosin VI motor activity was more directly studied by measuring in vitro actin sliding activity. Fig. 11A shows the effect of Thr$^{406}$ phosphorylation by PAK3KD on the motility activity of myosin VI. There was little motility activity of M6HMM before incubation with PAK3KD, while a significant amount of F-actin filaments moved with the velocity of 0.4 ± 0.1 $\mu M/s$ after incubation of M6HMM with PAK3KD. The addition of stauro-
sporine, a nonselective protein kinase inhibitor, significantly attenuated the PAK3KD-induced activation of the motility activity. The result suggested that PAK3KD-induced phosphorylation activated the motility activity of M6HMM. To evaluate this view, the phosphorylation level of M6HMM at Thr406 was monitored by Western blot using anti-phosphorylated Thr406-specific antibodies. There was a trace amount of the phosphorylated M6HMM observed before incubation with PAK3K, while a significant amount of phosphorylation was observed after incubation of M6HMM with PAK3KD. The addition of staurosporine significantly diminished the phosphorylation, although it did not completely abolish the phosphorylation. These results clearly indicated that the phosphorylation of M6HMM at Thr406 activates the motility activity of myosin VI. Interestingly, T406A mutant as well as T406E mutant showed in vitro motility activity similar to that of the phosphorylated wild type M6HMM (not shown). It has been shown that the mutation of the phosphorylatable Ser to Ala mimics the phosphorylated state of the protein rather than the dephosphorylated state, suggesting that the hydroxyl side chain of Ser plays a critical role in the dephosphorylated state (40). The present study provides another example of the Ala mutation mimicking the phosphorylated state.

It should be mentioned that a few F-actin filaments moved on M6HMM even before incubation with PAK3KD with similar velocity to the PAK3KD-phosphorylated M6HMM. But this is likely to be due to the presence of some prephosphorylated myosin VI, and there was a trace amount of phosphorylated M6HMM before incubation with PAK3KD (Fig. 11B). Consistently, it was found that the M6HMM preparation contains some trace myosin VI kinase activity (not shown).

**DISCUSSION**

During the last decade, a number of new myosins have been found that lack thick filament forming ability and are thought to play a role in diverse cellular contractile/motile functions. A key issue is how these myosins determine their specific role in a particular cellular function. One aspect of this issue involves the characteristics of motor activity such as processivity, actin translocating velocity, force, and directionality of movement. In this regard, myosin VI is a unique motor that moves on actin filaments toward the minus end (12) in a manner opposite to other known myosin family members. As a second factor, there may be a myosin-specific targeting site that determines each myosin’s cellular binding partner and subcellular localization. The tail portion of each myosin is a good candidate region for

![Figure 9](image1.png) **Fig. 9.** Binding of acto-M6HMM in the presence of ATP. The co-sedimentation assay was performed as follows. M6HMM (0.1 mg/ml) was incubated in buffer containing 20 mM imidazole, pH 7.5, 50 mM KCl, 2 mM ATP, 3 mM MgCl2, and various concentrations of F-actin in the presence of an ATP regeneration system (20 units/ml pyruvate kinase and 3 mM phosphoenolpyruvate) at 25 °C for 30 min. The samples were ultracentrifuged at 100,000 × g for 30 min, and the pellets were analyzed by SDS-polyacrylamide gel electrophoresis. The amount of the co-sedimented heavy chain was determined by densitometry as described previously (20).

![Figure 10](image2.png) **Fig. 10.** Effect of free Ca2+ on the actin filament sliding velocity of M6HMM. M6HMM was phosphorylated with PAK3 in a buffer containing 25 mM NaCl, 50 mM imidazole-HCl (pH 7.5), 10 mM MgCl2, 0.3 mM dithiothreitol, 0.1 μM microcystin, and 0.5 mM ATP at 25 °C for 15 min prior to use in the in vitro motility assay. Actin filament motility was observed in 30 mM KCl, 25 mM imidazole-HCl (pH 7.5), 5 mM MgCl2, 4.4 mg/ml glucose, 216 μg/ml glucose oxidase, 36 μg/ml catalase, 0.1 mM dithiothreitol, 0.1 mM microcystin, 2 mM ATP, and various concentrations of free Ca2+ at 25 °C. The flow cells with M6HMM filled with pCa5 buffer were refilled with EGTA buffer for restoration of motility. Measurements were made with 17–29 actin filaments to obtain an average velocity for preparation. All values are mean velocity ± S.D.

![Figure 11](image3.png) **Fig. 11.** Effect of PAK3 on Thr406 phosphorylation and actin movement in the in vitro motility assay of M6HMM. M6HMM was incubated with or without PAK3 (0.2 mg/ml) in a buffer containing 25 mM NaCl, 50 mM imidazole-HCl (pH 7.5), 10 mM MgCl2, 0.3 mM dithiothreitol, 0.1 μM microcystin, and 0.5 mM ATP at 25 °C for 15 min. Staurosporine (1, 10, and 100 μM) was added as indicated. The samples were subjected to in vitro motility assay and Western blot analysis. A, fraction of moving actin filaments. Actin filament motility was determined as described in the legend to Fig. 10, except 5 mM ATP and 1 mM EGTA were used. The probability of moving actin was determined by the ratio of moving filaments to whole actin filaments (number of 233–389 filaments) observed. B, Western blot analysis of M6HMM with anti-phospho-Thr406-specific antibodies.
achieving this specificity, since the structure of this domain is quite diverse. The third factor determining cellular specificity of myosin function is that the regulatory cascades that turn each myosin’s activity on could also be distinct, thus allowing separate activation of the different types of myosin. However, little is known for the regulatory mechanism of these unconventional myosins. The aim of this study is to clarify the motor function and regulation of mammalian class VI myosin.

The present study revealed a dual regulation of myosin VI by PAK3-induced phosphorylation and by Ca\(^{2+}\) binding to calmodulin. The former is required for the activation of myosin VI motility activity, and the latter inhibits the motility activity. The phosphorylation of myosin VI was previously observed with a bacteria-expressed glutathione S-transferase fusion fragment (amino acids 308–631) of myosin VI (41), but the present study is the first to identify the phosphorylation site as Thr\(^{406}\), because 1) the anti-phospho-Thr\(^{406}\)-specific antibodies recognized myosin VI phosphorylated by PAK3 and 2) \(^{32}\)P incorporation was markedly attenuated by the mutation of Thr\(^{406}\) to Ala.

We concluded that the phosphorylation of myosin VI is required for the activation of the motility activity of myosin VI, based upon the following results: 1) the incubation of myosin VI with PAK3 significantly increased the number of moving actin filaments, and this is accompanied by the increase in Thr\(^{406}\) phosphorylation, and 2) a protein kinase inhibitor, staurosporine, significantly attenuated the number of sliding actin filaments, and this is correlated with the decrease in the phosphorylation at Thr\(^{406}\). It should be mentioned that a few actin filaments moved without prephosphorylation with PAK3, but this is likely to be due to the presence of a low amount of phosphorylated myosin VI. The amount of prephosphorylated myosin VI varied from preparation to preparation, and we have prepared M6HMM that is entirely inactive in actin sliding activity without phosphorylation by PAK3. The results indicate that the phosphorylation of myosin VI is critical for the activation of the motility activity.

The phosphorylation of an analogous site has been reported for amoeba myosin I (24). In the case of amoeba myosin I, the phosphorylation is required for the actin activation of the ATPase activity. This is quite different from the regulation of myosin VI, in which the phosphorylation is necessary for the motility activity but not the actin-activated ATPase activity. The apparent mechanism for the uncoupling between the ATP hydrolysis and the motility is unknown. It has been suggested that a change in the rigidity of the lever arm domain of myosin could uncouple the motility and ATPase activity of myosin (42); however, this is less likely, since the location of Thr\(^{406}\) is near the tip of the head of myosin. Another possibility is a change in the fraction of the strong and weak binding states induced by phosphorylation that is in turn related to the “processivity” of myosin. It has been shown recently that vertebrate myosin V is a processive motor (43, 44) and that the processivity is closely related to the identity of the stable intermediate of the myosin V ATPase reaction as myosin:ADP, a strong actin binding intermediate (38). This is also less likely, since during the ATPase reaction the apparent affinities (K\(_{\text{actin}}\)) for actin of phosphorylated and dephosphorylated myosin VI are similar to each other. The mechanism by which phosphorylation activates the motility activity of myosin VI requires further investigation.

While the phosphorylation is necessary for the activation of the motility activity of myosin VI, Ca\(^{2+}\) inhibited the motility activity. The loss of motility at high Ca\(^{2+}\) was not due to the denaturation of myosin VI, since perfusion of the flow cell with low Ca\(^{2+}\) buffer (below pCa 7) completely restored the motility (Fig. 10). We examined the effect of various exogenous calmodulin concentrations up to 12 \(\mu\)M; therefore, it is unlikely that the loss of motility is due to unsaturation of bound calmodulin. The Ca\(^{2+}\)-dependent inhibition of mammalian unconventional myosin was first demonstrated with myosin I (17–21) and then subsequently with myosin V (22, 23). It was thought originally that the inhibition of motility was due to the dissociation of calmodulin from the heavy chain at high Ca\(^{2+}\) (17, 18). However, recent studies have revealed that the inhibition does not require the dissociation of calmodulin, but rather Ca\(^{2+}\) binding at the high affinity sites of calmodulin triggers the inhibition presumably due to a large conformational change of calmodulin (20–22). For myosin V, Ca\(^{2+}\)-dependent regulation requires at least two calmodulin binding sites (22, 23), and a truncated mutant having a single calmodulin was constitutively active (22, 23). Interestingly, the present study revealed that myosin VI, also binding a single calmodulin, is regulated by Ca\(^{2+}\). The bound calmodulin was not dissociated from the heavy chain at high Ca\(^{2+}\); nevertheless, myosin VI motility was inhibited. This is consistent with the most recent results with myosin I\(\beta\) and myosin V and supports the idea that calmodulin dissociation is not the inhibitory mechanism of calmodulin binding myosins. The reason why the IQ/calmodulin of myosin VI, but not the corresponding first IQ/calmodulin of myosin V, plays a role in the regulation of motility is unclear. But it is plausible that the position of the first calmodulin of myosin VI resembles the second calmodulin site for myosin V, since a unique large insert is present between the converter and the IQ domain of myosin VI.

The inhibition occurs between pCa 7 and pCa 6 where cytoplasmic Ca\(^{2+}\) concentration is regulated in most cell types, and therefore the observed inhibition is physiologically relevant. Since this range of Ca\(^{2+}\) concentrations corresponds to the high affinity C-terminal sites of calmodulin, it is likely that Ca\(^{2+}\) binding to the C-terminal lobe of calmodulin and consequent conformational changes are responsible for the inhibition of motility. Supporting this notion, it was shown that abolition of the C-terminal Ca\(^{2+}\) binding sites abolishes inhibition of the motility of myosin I\(\beta\) (21).

There is an apparent decoupling between the ATP hydrolysis cycle and mechanical events at higher Ca\(^{2+}\). One possibility is a change in the “processivity” of myosin VI by Ca\(^{2+}\), but this is not the case, since Ca\(^{2+}\) did not affect the K\(_{\text{actin}}\) of the actin-activated ATPase reaction. This is different from the regulation of conventional myosins in which the regulatory domain regulates both the ATPase and mechanical activities (13–15). It is plausible that the change in calmodulin conformation alters the rigidity of the “lever arm” and thus decouples the chemical and mechanical events. Alternatively, the conformational change of calmodulin could alter the interaction between calmodulin and the “converter” domain of myosin VI, thus inhibiting motility.

A question is whether myosin VI is a processive motor. As shown in Fig. 9, myosin VI strongly binds to actin in the presence of Mg\(^{2+}\)-ATP. It is also revealed that the actin-activated ATPase activity is markedly decreased with time, and this is due to the ADP produced during the course of myosin VI ATPase reaction, because the decrease in the activity with time is completely eliminated in the presence of an ATP regeneration system. The result suggests that myosin VI has a strong ADP binding relative to the ATP binding. Consistently, K\(_{\text{ATP}}\) was extremely high for the myosin VI ATPase reaction (150 \(\mu\)M), and a much lower K\(_{\text{ADP}}\) value (3 \(\mu\)M) is estimated. This result is consistent with the strong actin binding nature of myosin VI in the presence of Mg\(^{2+}\)-ATP and suggests that the ADP-bound form (i.e. a strong actin binding state) is the pre-
dominant steady state intermediate of myosin VI ATPase cycle. It should be noted that even at low phosphorylation level, a few actin filaments moved with a velocity similar to that of the highly phosphorylated myosin VI. The result suggests that the active form of myosin VI can support the actin filament sliding at very low surface density of the active myosin molecules. These properties of myosin VI are similar to those of myosin V, a processive motor (38, 43, 44). Therefore, it is anticipated that myosin VI has a processive nature in its motor function, although more direct evidence is required to determine the processivity of myosin VI.

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Dual Regulation of Mammalian Myosin VI Motor Function
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