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Enzymatic Characterization and Functional Domain Mapping of Brain Myosin-V*

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The actin binding and ATPase properties, as well as the functional domain structure of chick brain myosin-V, a two-headed, unconventional myosin, is reported here. Compared to conventional myosin from skeletal muscle, brain myosin-V exhibits low K-EDTA- and Ca-ATPase activities (1.8 and 0.8 ATP/s per head). The physiologically relevant Mg-ATPase is also low (~0.3 ATP/s), unless activated by the presence of both F-actin and Ca^{2+} (V_{max} of 27 ATP/s). Ca^{2+} stimulates the actin-activated Mg-ATPase over a narrow concentration range between 1 and 3 μM. In the presence of saturating Ca^{2+} and 75 mM KCl, surprisingly low concentrations of F-actin activate the Mg-ATPase in a hyperbolic manner (K_{ATPase} of 1.3 μM). Brain myosin-V also binds with relatively high affinity (compared to other known myosins) to F-actin in the presence of ATP, as assayed by cosedimentation. Digestion of brain myosin-V with calpain yielded a 65-kDa head domain fragment that cosediments with actin in an ATP-sensitive manner and a 80-kDa tail fragment that does not interact with F-actin. The 80-kDa fragment results from cleavage one residue beyond the proline-, glutamate-, serine-, threonine-rich region. Our data indicate that the Mg-ATPase cycle of brain myosin-V is tightly regulated by Ca^{2+}, probably via direct binding to the calmodulin light chains in the neck domain, which like brush border myosin-I, results in partial (~30%) dissociation of the calmodulin associated with brain myosin-V. The effect of Ca^{2+} binding, which appears to relieve suppression by the neck domain, can be mimicked by calpain cleavage near the head/neck junction.

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Although the conventional myosins (myosins-II) responsible for processes such as muscle contraction and cytokinesis have been intensively studied, relatively little is known about the properties of the unconventional myosins. One widely distributed group of unconventional myosins, the class V myosins, have been suggested to play a role in organelle transport or membrane targeting (reviewed in Refs. 1 and 2). Brain myosin-V (BM-V[1]), which was originally identified as a calmodulin (CaM)-binding protein in vertebrate brain, is the only member of this class of unconventional myosins to be purified and characterized biochemically (3–7). The class V myosins share a common domain structure consisting of an N-terminal head domain containing the actin-binding and ATP hydrolysis sites, an extended neck domain containing six IQ motifs (which form binding sites for CaM and/or related light chains), and a tail domain consisting of a region predicted to form coiled coils attached to a globular region of unknown function (7, 8). The hypothetical functions of the class V myosins and their novel domain structure, particularly the extended neck domain, raise the obvious question of how the basic biochemical properties of the class V myosins compare with those of other types of myosins.

Among the critically important properties of a molecular motor are its steady-state ATPase activity and the factors which regulate this activity. Known myosins are regulated either indirectly via actin-associated proteins such as troponin/tropomyosin (as in vertebrate skeletal muscle myosin) or directly via the subunits of the myosin molecule itself. In the latter type of direct "myosin-linked" regulation, the light chains associated with the neck domain often function as the regulating subunits. The myosin light chains are all members of the CaM superfamily of proteins, although not all of them have retained the ability to bind to Ca^{2+}. Vertebrate non-muscle and smooth muscle myosins-II are "turned on" by phosphorylation of their regulatory light chains by Ca^{2+}/CaM-dependent myosin light chain kinase (9), whereas molluscan myosin-II and vertebrate brush border (BB) myosin-I are regulated by the direct binding of Ca^{2+} to their light chains (10, 11). Like BB myosin-I, BM-V has multiple CaM light chains that remain bound in the absence of Ca^{2+} (3, 5, 7). The neck domain of BM-V has been shown to be the precise site of CaM binding (8), suggesting that this myosin might also be directly regulated by Ca^{2+} binding. The initial reports of BM-V ATPase activity also indicated that this protein is activated by Ca^{2+} (5, 7), although the K_{ATPase} for Ca^{2+} and F-actin were not determined.

The tail domains of myosins are unique to each class in the myosin superfamily and probably reflect the specific functions

[1] The abbreviations used are: BM-V, brain myosin-V; CaM, calmodulin; BB, brush border; PEST, proline, glutamate, serine, threonine rich; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

and/or targets for each individual myosin (1). Although the proximal region of the BM-V tail appears to lead to dimer formation via coiled coil segments, BM-V does not form myosin filaments (7). The medial region of the BM-V tail contains a PEST (proline-, glutamate-, serine-, threonine-rich) region (8), a sequence motif which has been hypothesized to be associated with intracellular proteolysis by calpain (12, 13). BM-V is, in fact, a substrate for limited proteolysis by calpain (5, 6), which results in a stable 65-kDa head fragment and a stable 80-kDa tail fragment. The function of the globular C-terminal tail domain that characterizes the class V myosins is unknown, although this domain shares sequence homology with the human protein AF-6 and the related *Drosophila* protein, canoe (14).

In the present study, we have determined the ATPase properties of highly purified BM-V from freshly hatched chicks and demonstrate that the actin-activated Mg-ATPase activity is tightly regulated by physiologically relevant concentrations of Ca^{2+}. The maximal actin-activated Mg-ATPase of BM-V is quite high and is comparable to that of skeletal muscle myosin. Surprisingly, however, myosin-V is activated by much lower concentrations of F-actin than is skeletal muscle myosin. We have taken advantage of the cleavage of BM-V by calpain to obtain information on the functions and interactions of the domains of BM-V. Our data indicate that, similar to other regulated myosins, the neck domain of BM-V has a suppressive effect on the ATP-hydrolysis/actin-binding cycle of the head domain. This suppression can be relieved by binding to Ca^{2+} or by specific cleavage near the head/neck junction. We have also localized one of the two major calpain cleavage sites in BM-V to a site in the tail just one amino acid downstream from the PEST region. The 80-kDa tail fragment generated by this cleavage includes the globular domain of unknown function. This domain did not bind to actin in cosedimentation assays and does not appear to contain a second actin-binding site.

EXPERIMENTAL PROCEDURES

Materials—Electrophoresis chemicals, molecular mass standards, imidazole, ATP (grade II), EDTA, EGTA, dithiothreitol (DTT), aprotinin, benzamidine, bovine brain CaM, and rabbit skeletal muscle calpain were purchased from Sigma. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were obtained from Sigma or Promega (Madison, WI). Anti-rabbit and anti-mouse IgGs conjugated to alkaline phosphatase were from Promega. Pefabloc, calpain inhibitor I, and phalloidin were from Boehringer Mannheim. Chromatography media were from Pharmacia Biotech Inc.

Proteins—Myosin-V was purified from chick brain essentially as described by Cheney *et al.* (7), except for a change in the initial centrifugations from 18,000 to 40,000 × *g* and the addition of the protease inhibitors Pefabloc (1 mM), benzamidine (1 mM), and aprotinin (2 μg/ml). These modifications and improved assay conditions resulted in higher yields and higher maximum ATPase activity than previously obtained. Actin was purified from chicken pectoralis muscle by the method of Spudich and Watt (15). Affinity-purified antibodies to the myosin-V tail ("tail antibody"; Ref. 8) and a monoclonal antibody, MaB CX-1, made to BB myosin-I that reacts with the N-terminal third of the BB myosin-I head as well as with BM-V ("head antibody"; Ref. 16) were used for immunoblot analysis.

ATPase Assays—ATPase activity was assayed at 37 °C using the colorimetric method of Taussky and Shorr (17). The K-EDTA-, Ca-, and Mg-ATPase activities of 0.06 μM BM-V were measured over 15 min; actin-activated Mg-ATPase activities of 0.02 μM BM-V were determined for 5-min reactions. The hydrolysis of ATP, expressed as ATP/s per myosin head, was calculated by considering the molecular mass of native myosin-V dimers to be 640 kDa (7). The final conditions used in the assays were as follows: K-EDTA-ATPase, 20 mM imidazole, pH 7.4, containing 75 or 600 mM KCl, 5 mM EDTA, and 2 mM DTT; Ca-ATPase, 20 mM imidazole, pH 7.4, containing 75 mM KCl, 5 mM $CaCl_2$, and 2 mM DTT; Mg-ATPase, 20 mM imidazole, pH 7.4, containing 75 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and 2 mM DTT; and actin-activated Mg-ATPase, 20 mM imidazole, pH 7.4, containing 75 mM KCl, 2.5 mM $MgCl_2$, 2 mM DTT, and actin concentration as indicated. The effect of Ca^{2+} on the actin-activated Mg-ATPase activity was assessed by the addition of 4 mM Ca-EGTA buffers to the standard assay, covering a range of free calcium from 10^{-7} to 10^{-4} M. Free calcium was calculated by the Mcalc program of Fabiato (18), using the Schwartzenbach dissociation constants for Ca-EGTA. The dependence of Mg-ATPase activity on actin concentration was determined by addition of F-actin over a range of concentrations from 0.1–60 μM. Phalloidin (1:1 molar ratio with actin) was added to solutions containing 0.1 to 10 μM actin to stabilize the actin as filaments. The ATPase activity of F-actin alone was measured at each concentration under the same reaction conditions, and its contribution was subtracted from the corresponding actin-activated activity of BM-V.

Actin Binding Assays—The binding of BM-V to F-actin in the presence of ATP was assessed using assay conditions identical to those used for the Mg-ATPase assays, except that the Mg-ATP concentration was 10 mM and the pH was 7.1. BM-V (0.03 μM) was mixed with phalloidin-stabilized F-actin at 0, 0.5, 1.0, 5.0, and 10 μM, in either 4 mM EGTA or 4 mM Ca^{2+}/EGTA buffer (4 mM $CaCl_2$, 4 mM EGTA; ~11 μM free Ca^{2+}), and then centrifuged for 1 h at 100,000 × *g*. The resulting pellet and supernatant fractions were analyzed by SDS-PAGE, and the percentage of BM-V cosedimented with F-actin was determined by densitometry.

Ca^{2+}-dependent Dissociation of CaM from BM-V—The effect of Ca^{2+} on CaM binding to BM-V heavy chain was assessed by the method of Wolenski *et al.* (19). Aliquots of BM-V (0.16 μM) were incubated at room temperature for 15 min in 75 mM KCl, 2.5 mM $MgCl_2$, 10 mM imidazole, pH 7.1, 0.5 mM DTT, and either 1 mM $CaCl_2$ or 4 mM Ca^{2+}/EGTA buffers giving free [Ca^{2+}] of ~1.5, 5, and 25 μM ($CaCl_2$:EGTA ratios of 0:1, 0.9:1, 0.95:1, and 1:1). Bound and free CaM light chains were separated by actin cosedimentation (100,000 × *g* for 1 h). F-actin (9 μM) was added, and the samples were centrifuged at 100,000 × *g* for 1 h. The pellet (containing BM-V-associated CaM light chains) and supernatant (free CaM) were analyzed by SDS-PAGE and densitometry.

Calpain Digestion of BM-V—Proteolytic digestions of BM-V were performed using a 1:80 ratio (w/w) of calpain to BM-V. Digestion were allowed to proceed for 30 s to 2 h at 25 °C in the presence of 2 mM $CaCl_2$ and 2 mM DTT. The reactions were quenched by the addition of 5 mM EGTA or calpain inhibitor I to 1 μM. Samples were subsequently assayed for actin-activated Mg-ATPase activity and analyzed by SDS-PAGE and immunoblot. The binding of calpain-generated fragments to actin was analyzed by incubating F-actin (9 μM) and BM-V (0.1 μM) or fragments in 10 mM imidazole, pH 7.4, containing 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EGTA, and 2 mM DTT, in the presence or absence of 2 mM ATP, for 10 min at room temperature. The samples were then centrifuged at 100,000 × *g* for 15 min in a Beckman Airfuge (Palo Alto, CA). The protein composition of the resulting supernatant and pellet fractions was analyzed by SDS-PAGE.

Identification of the Primary Calpain Cleavage Site on BM-V—The 80-kDa fragment, generated by calpain cleavage of 0.5 mg of BM-V, was purified by anion exchange chromatography on a 1-ml Q-Sepharose column equilibrated in 10 mM imidazole, pH 7.4, containing 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EGTA, and 2 mM DTT. Fractions (0.5 ml) were collected during elution of the column with 30 ml of 75–1000 mM KCl gradient in column buffer, at a flow rate of 10 ml/h, and analyzed by SDS-PAGE. Those containing the pure 80-kDa fragment were pooled, precipitated with ice-cold 5% trichloroacetic acid, and submitted to N-terminal microsequencing at the Yale University W. M. Keck Foundation Biotechnology Resource Laboratory.

Amino Acid Analysis of 65- and 80-kDa Fragments of BM-V—The 65- and 80-kDa fragments, generated by calpain cleavage of BM-V, were purified by reverse phase high-performance liquid chromatography on a C_4 column (Vydac, Hesperia, CA) using a 12–80% acetonitrile gradient in 0.1% trifluoroacetic acid. Approximately 860 ng of the 65-kDa and 970 ng of the 80-kDa fragments were vacuum dried, hydrolyzed in 6 N HCl, derivatized with phenyl isothiocyanate, and analyzed according to Bidlingmeyer and Tarvin (20) on a model MP3000E binary system from Laboratory Data Control (Milton Roy Co., Riviera Beach, FL).

Other Methods—SDS-PAGE was performed using 5–16% linear gradient minigels. Immunoblot analysis was performed as described by Towbin *et al.* (21). Concentrations of purified proteins were calculated based on the following extinction coefficients at 280 nm for 1.0 mg/ml solutions (7): BM-V, 1.04; and actin, 1.09. Protein concentrations of mixtures were determined by the method of Bradford (22).

RESULTS

ATPase Activities of Purified BM-V—Since myosins generally have little basal ATPase activity in the presence of physiological concentrations of Mg^{2+}, they are often assayed under

TABLE I
ATPase activities of brain myosin-V: K-EDTA-, Ca-, and Mg-ATPase

Samples containing myosin-V at 0.06 μM were incubated for 15 min at 37 °C. ATPase activity was assayed as described under "Experimental Procedures." Values reported are the average specific activities and S.D. from the same preparation (n = 3). Similar results were obtained for different preparations of myosin-V.

Assay condition	ATPase activity (ATP/s per head)		
	K^+-EDTA	Ca^{+2}	Mg^{+2}
600 mM KCl	1.77 (±0.07)		
75 mM KCl	0.55 (±0.01)	0.76 (±0.07)	0.27 (±0.03)

conditions that displace Mg^{2+} from the protein. Traditionally, this has meant assaying in the presence of EDTA and ~0.6 M KCl. Although these K-EDTA-ATPase conditions are clearly not physiological, they have historically provided a simple test for myosin-like activity. Under these conditions, BM-V exhibits a modest ATPase of 1.8 ATP/s per head (Table I). This K-EDTA-ATPase activity is similar to that reported for BB myosin-I (2.3 ATP/s; Ref. 23) but lower than those reported for skeletal muscle myosin-II (~10 ATP/s per head for the HMM fraction; Ref. 24) and *Acanthamoeba* myosins-I (12–22 ATP/s; Ref. 25). Because BM-V does not form filaments, it can also be assayed at lower salt concentrations (75 mM KCl), where its K-EDTA-ATPase is ~0.5 ATP/s (Table I). Most importantly, BM-V exhibited little or no basal Mg-ATPase activity (0–0.3 ATP/s, depending on the preparation).

Ca^{2+} Tightly Regulates the Actin-activated Mg^{2+}-ATPase Activity of BM-V—The ATPase values reported in Table I are strikingly low, especially when compared with the Mg-ATPase observed in the presence of both F-actin and Ca^{2+} (Fig. 1). BM-V exhibits little or no actin-activated Mg-ATPase activity in the absence of Ca^{2+}. Even in the presence of Ca^{2+}, BM-V by itself does not manifest substantial Mg-ATPase activity. Only if both F-actin and Ca^{2+} are present is ATP rapidly hydrolyzed. These data show that BM-V is tightly regulated by Ca^{2+} in a manner reminiscent of molluscan myosin-II (26) and vertebrate myosin-I (19, 23, 27, 28).

The actin-activated Mg-ATPase activity of BM-V is further stimulated by the addition of bovine brain CaM (Fig. 1). The degree of this stimulation varies somewhat from preparation to preparation, perhaps due to loss of CaM during purification or storage. Since the addition of higher concentrations of CaM did not lead to further increases in ATPase activity (data not shown), 5.9 μM CaM was included in all subsequent Mg-ATPase assays to guarantee maximal activity.

Regulation of the Actin-activated ATPase Occurs over a Narrow Range of Ca^{2+} Concentration—The effect of Ca^{2+} on the actin-activated Mg-ATPase activity of BM-V is shown in Fig. 2. The activation occurs over a very narrow range of Ca^{2+} concentration, with no significant effect detected up to about 1 μM and a near maximum effect at about 3 μM Ca^{2+} (50% activation at 1.8 μM Ca^{2+}). Although there are few data points at the critical inflection region, a Hill plot of the available data yielded an n value of 13, suggesting a high degree of cooperativity. Even at concentrations of Ca^{2+} up to 1 mM, BM-V maintained the high rate of ATP turnover (data not shown). To determine whether this range of Ca^{2+} results in dissociation of CaM from BM-V, as has been observed for BB myosin-I (11), two different preparations of BM-V were used to examine the effect of elevated Ca^{2+} on CaM light chain content. Using actin cosedimentation to separate heavy chain bound and free CaM light chains (19), we observed partial dissociation of CaM (27–32%; 1–1.3 CaM/heavy chain) at Ca^{2+} concentrations above 5 μM, with no further increase even at 1 mM Ca^{2+}.

BM-V Is Activated at Low Actin Concentrations and Hydrolyses ATP at a Fast Rate—We determined the effect of the actin concentration on the Mg-ATPase activity in the presence of saturating Ca^{2+}. F-actin activates the Mg-ATPase activity in a simple hyperbolic manner (Fig. 3*A*). The dependence of the Mg-ATPase activity on actin concentration was also assayed at various myosin concentrations (data not shown). We observed no evidence of the triphasic activation seen in the *Acanthamoeba* myosin-I, which is due to the presence of a second actin-binding site in the tail domain (25). A double reciprocal

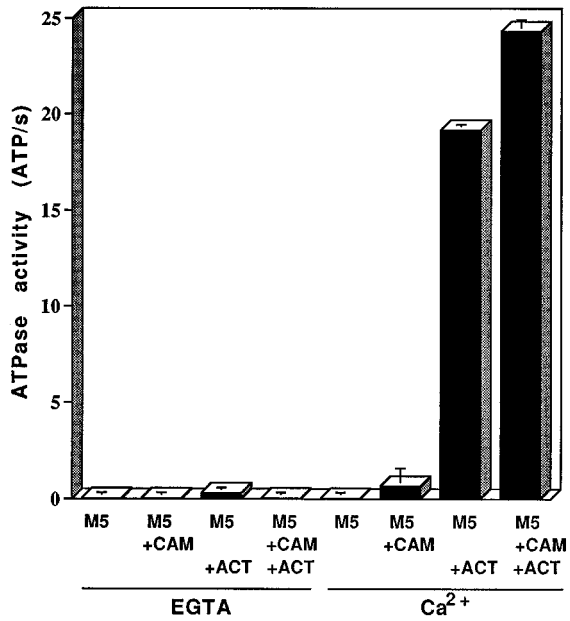

FIG. 1. **The high Mg^{2+}-ATPase activity of BM-V requires both Ca^{2+} and actin.** The Mg-ATPase activity of BM-V (0.02 μM) was assayed as described under "Experimental Procedures" in the presence of 75 mM KCl and 4 mM EGTA, 0.5 mM $CaCl_2$, 5.9 μM additional CaM (*CAM*), and/or 20 μM F-actin (*ACT*), as indicated. The data presented is from a single preparation assayed in triplicate. Similar results were obtained from other preparations, although the actin-activated Mg-ATPase in the absence of Ca^{2+} exhibited some variation (0–3 ATP/s), as did the basal Mg-ATPase activity in the presence of Ca^{2+} (0–2 ATP/s).

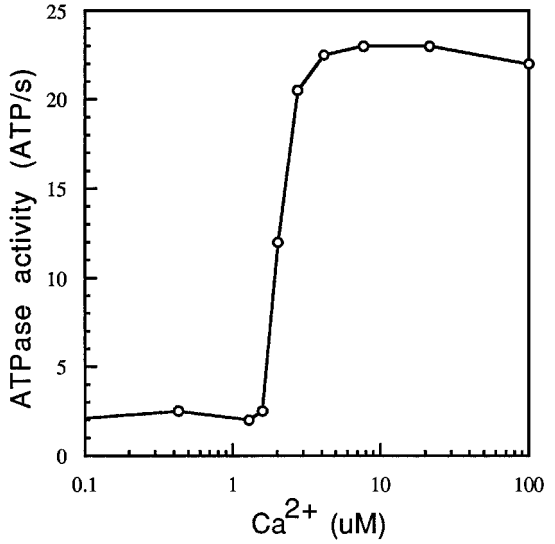

FIG. 2. **Ca^{2+} in the micromolar range stimulates the actin-activated ATPase activity of BM-V.** The actin-activated Mg-ATPase activity of BM-V (0.02 μM) was assayed in the presence of 75 mM KCl, 20 μM F-actin, 5.9 μM additional CaM, and free Ca^{2+} concentrations, as indicated, using a Ca-EGTA buffer system. The value in EGTA alone was 2 ATP/s. The data presented are from a single preparation assayed in triplicate; *bars*, S.D. Similar results were obtained from other preparations.

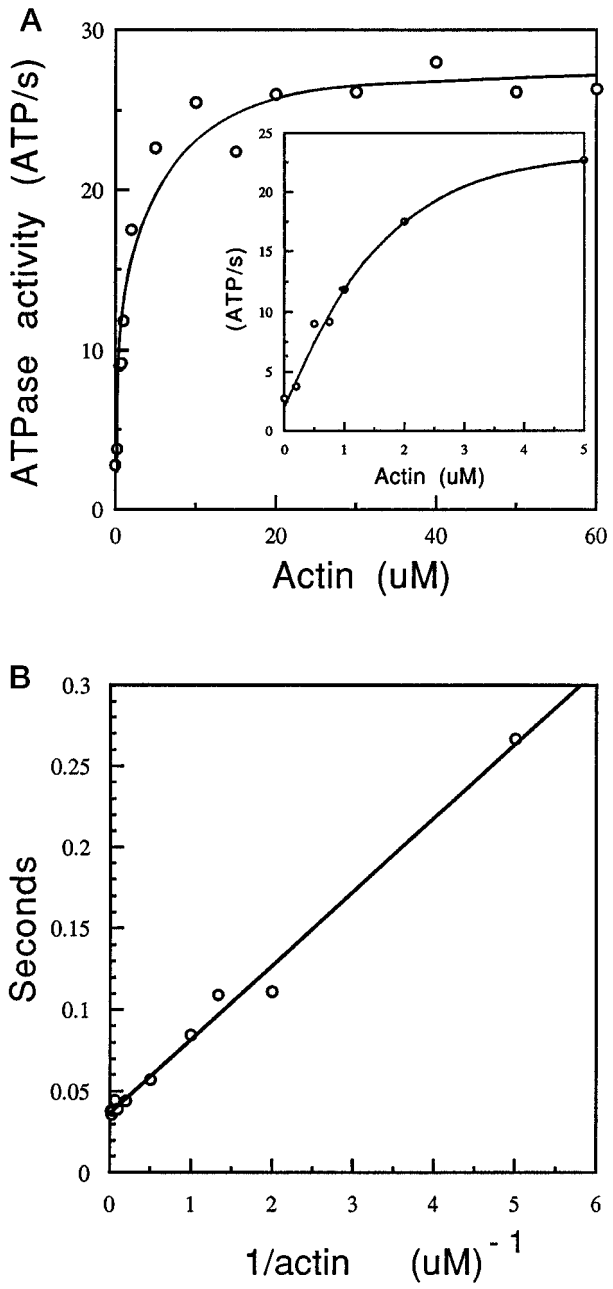

FIG. 3. **Low concentrations of F-actin activate the Mg^{2+}-ATPase activity of BM-V.** The effect of actin concentration on the Mg-ATPase activity of BM-V (0.02 μM) was assayed in the presence of 75 mM KCl, 0.5 mM $CaCl_2$, and 5.9 μM additional CaM. *A*, ATP turnover as a function of actin concentration. The *inset* shows an expansion of the data points at low actin concentrations. *B*, double reciprocal plot of the data to yield the V_{max} (27 ATP/s) and K_{ATPase} (1.3 μM). Different preparations of BM-V gave K_{ATPase} values that ranged from 1 to 2 μM.

plot of the BM-V data (Fig. 3*B*) yielded a V_{max} of 27 ATP/s per myosin head and a K_{ATPase} of 1.3 μM. This value for maximal actin-activated Mg-ATPase activity puts BM-V amongst the enzymatically fastest myosins, including skeletal muscle myosin-II (24), molluscan myosin-II (26), and amoeba myosin-I (25, 29, 30). Interestingly, however, the K_{ATPase} value suggests that BM-V binds much more tightly to F-actin than these other myosins. Consistent with this finding, BM-V exhibits relatively high levels (compared to other known myosins) of binding to actin in the presence of ATP, particularly in the presence of Ca^{2+} (Fig. 4), as assessed by cosedimentation with F-actin under solution conditions comparable to those used in the Mg-ATPase assay, except that the Mg-ATP concentration was 10 mM (to insure that ATP depletion did not occur). Comparable results to those summarized in Fig. 4 were seen in the presence of exogenously added CaM (results not shown). This binding is actin concentration-dependent, whereas no such dependence can be detected for binding under rigorous conditions, where >90% pelleting of BM-V is observed at all actin concentrations tested (results not shown).

Calpain Cleaves BM-V at the PEST Site in the Tail and near the Head/Neck Junction—We have previously shown that the calcium-dependent protease calpain cleaves BM-V in a limited manner *in vitro* and generates two stable polypeptides with apparent molecular masses of 65 and 80 kDa, which correspond to regions of the head and tail of BM-V, respectively (5, 6). In the present study, the time course of proteolysis of purified BM-V by calpain was determined (Fig. 5*A*). BM-V was rapidly cleaved by calpain generating 80- and 130-kDa fragments. The 130-kDa fragment was further proteolyzed to a 65-kDa fragment. Both the 130- and 65-kDa fragments were recognized by head antibodies (Fig. 5*B*), whereas the 80-kDa fragment was recognized only by tail antibodies (Fig. 5*C*). A diagram of the putative localization of these sites is shown in Fig. 5*D*.

The precise cleavage site that generates the 80-kDa fragment was determined by microsequencing of the N-terminal of the purified 80-kDa polypeptide (Fig. 5*E*) and is located between Arg^{1140} and Met^{1141}, just one amino acid downstream from the PEST site. Surprisingly, analysis of the microsequence data revealed the presence of two sequences in about equal amounts differing only by a single amino acid, the insert of a Gln as the third amino acid in one of the peptides (Fig. 5*E*). This same difference in a single amino acid appears in the two published primary sequences of chicken BM-V based on cDNA (Fig. 5*F*; Refs. 8 and 31) and thus may reflect different alleles or a single amino acid splicing variant.

Since the polypeptide corresponding to the C-terminal side of the second cleavage site has not been isolated, the exact site that generates the 65-kDa fragment has not yet been determined by microsequencing. We have, however, determined the approximate location of this cleavage site based on the apparent molecular mass in SDS-PAGE and a comparison of the amino acid composition of the purified 65-kDa fragment with the deduced amino acid composition of BM-V. This analysis, which is described in the legends of Table II and Fig. 6, suggests that the second cleavage site occurs near amino acid 629 ± 2 amino acids of BM-V (Fig. 6). Based on sequence alignment with skeletal myosin-II, this would place the cleavage site near the 50-kDa/20-kDa head domain junction in loop 2 of the actin-binding site (1, 8, 32).

The 65-kDa Head Fragment of BM-V, but not the 80-kDa Tail Fragment, Binds to F-Actin—The actin binding properties of the head and tail fragments of BM-V were assessed by actin cosedimentation in the absence and presence of ATP (Fig. 7). The 65-kDa head fragment exhibited ATP-dependent cosedimentation with F-actin. The 80-kDa fragment, on the other hand, remains in the supernatant under both conditions, indicating that it does not bind to actin filaments.

Calpain Proteolysis Deregulates the Actin-activated ATPase Activity of BM-V—After various incubation times with calpain, the actin-activated Mg-ATPase activity of BM-V was determined in the presence of 0.5 mM Ca^{2+} or 5 mM EGTA (Fig. 8). After proteolysis for 30–60 min, when intact BM-V was no longer detected, 65% of the actin-activated ATPase, originally Ca^{2+}-dependent, was retained. On the other hand, the Ca^{2+}-independent, actin-activated ATPase activity increased with time of proteolysis, eventually attaining rates of ATP turnover equal to those in the presence of Ca^{2+}. The low levels of ATPase

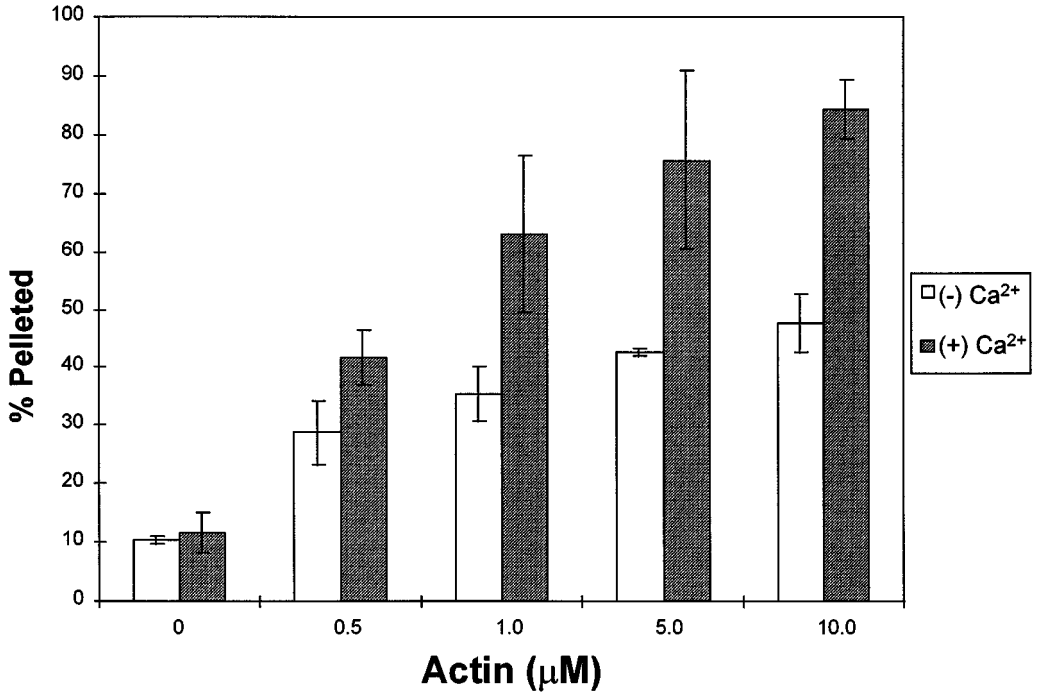

FIG. 4. **Cosedimentation of BM-V with actin in the presence of ATP.** This histogram plots the results of three separate experiments in which the percentage of total BM-V (0.03 μM) pelleted with F-actin (0–10 μM) in the presence of either 4 mM EGTA (□) or 10 μM free Ca^{2+} (▨). *Bars,* S.D.

activity observed in the absence of actin were not affected by proteolysis (data not shown). Thus, as seen in the insert to Fig. 8, cleavage by calpain leads to a loss of the Ca^{2+}-regulated ATPase activity while the actin-activated Mg-ATPase remains high. This "deregulation" is similar to that reported for molluscan myosin-II and BB myosin-I after proteolytic cleavage near their neck domains (10, 19).

DISCUSSION

The purification of myosin-V from vertebrate brain has allowed for the characterization of the mechanochemical properties and functional domain organization of now a third class amongst the 10–11 classes of the myosin superfamily of molecular motors (reviewed in Ref. 1). Thus, in addition to myosin-II and myosin-I, which have been extensively studied as native proteins, there is now enough biochemical data on BM-V to allow for an initial description of the domain functions and mechanochemical properties of a class V myosin, as well as a comparison with specific class I and class II myosins. Sequence analysis has shown that, although the different classes of myosins have strikingly different tail domains, their head domains are conserved in general structure and organization (1, 33, 34). Purified myosins, however, exhibit quantitative differences in mechanochemical properties, such as ATPase activity and sliding filament velocity, as well as substantial differences in regulation. It is now realized that all myosins contain a distinct neck domain responsible for light chain binding, which can have a regulatory role and perhaps also function as a lever for the generation of movement (33, 35–38). Because the neck domain of the class V myosins is much larger than the neck domain of the conventional myosins, there is much interest in comparing the mechanochemical properties of these myosins.

Mg^{2+}-ATPase Activity of BM-V—The results presented here indicate that the actin-activated Mg-ATPase of BM-V is comparable in rate to that of skeletal muscle myosin-II. However, a striking difference between skeletal muscle myosin and BM-V is that BM-V is activated by very low concentrations of F-actin. Since the ability of skeletal muscle myosin to bind to actin is greatly inhibited as the salt concentration increases, the magnitude of this difference may be even higher at truly physiological salt concentrations. Thus, BM-V appears to bind much more tightly to actin in the presence of ATP than does skeletal muscle myosin. Indeed, this binding was directly observed by cosedimentation of BM-V with F-actin under conditions comparable to those used in the ATPase assays (Fig. 4). Although Ca^{2+} enhances the binding of BM-V to actin in the presence of ATP, significant binding of BM-V to actin is seen in the absence of Ca^{2+} as well. Thus, the actin binding is much less stringent in its requirement for Ca^{2+} than the ATPase activation. This raises the possibility that the binding of BM-V to actin in the presence of ATP is mediated by a second, ATP-insensitive site, as observed in the tail domain of amoeboid myosins-I (25). This possibility cannot be ruled out by our present data. However, two lines of evidence suggest that this is not the case. (*a*) The actin activation of BM-V Mg-ATPase does not exhibit the triphasic behavior characteristic of amoeboid myosins-I. (*b*) Assessment of the actin binding properties of the calpain cleavage products of BM-V indicates that the 80-kDa tail fragment does not bind F-actin.

The activation of BM-V by low concentrations of actin and its relatively high affinity for actin in the presence of ATP both suggest that this myosin could function in regions of low actin filament concentration. This might be the case if, as has been suggested, BM-V functions as a motor for organelle transport.

BM-V Is Tightly Regulated by Ca^{2+} via a Myosin-linked System—The enzymatic studies presented here clearly show that BM-V is a regulated myosin. Maximum activation of Mg-ATPase activity occurred in the presence of about 20 μM purified skeletal muscle F-actin and 3 μM Ca^{2+}. Removing Ca^{2+} at the same actin concentration resulted in a greater than 90% inhibition of the ATPase activity. In fact, in most preparations, the degree of inhibition was closer to 99% (Figs. 1, 2, and 8). The concentration range of Ca^{2+} required to stimulate the ATPase is within a physiologically relevant realm, which suggests that Ca^{2+} may regulate the activity of this motor *in vivo.*

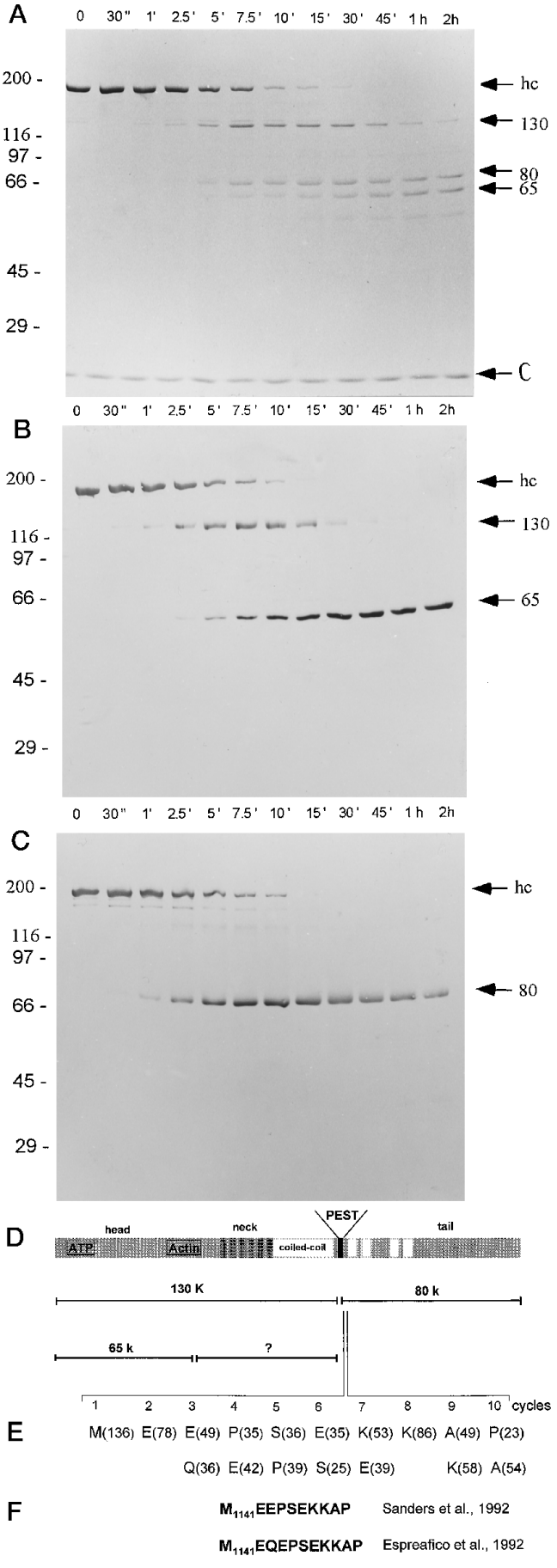

FIG. 5. **Time course of calpain digestion of BM-V and localization of the primary cleavage site.** Proteolytic digestion of BM-V was performed as described under "Experimental Procedures." At the times indicated, aliquots of the reaction mix were prepared for SDS-PAGE and immunoblots. *A*, Coomassie Blue-stained gel of these samples; *B*, Western blot of the same samples probed with the "head" antibody as described under "Experimental Procedures"; *C*, Western blot of the same samples probed with the myosin-V "tail" antibody. *hc*, the intact BM-V heavy chain. Polypeptides of 130, 80, and 65 kDa are indicated to the *right* of the figure, and positions of molecular mass standards are indicated to the *left*. A diagram of the fragments generated by calpain on BM-V is shown in *D*. The prominent 2 amino acids of each of 10 cycles of microsequence data from the N-terminal of the 80-kDa fragment is shown in *E*. Where only one amino acid is given, that amino acid was more than three times more abundant than any other amino acid at that cycle. pmol values are given in *parentheses*. The corresponding sequences derived from cDNA sequencing from two laboratories is given in *F*.

Although we have previously shown that the tail domain of BM-V is a substrate for Ca^{2+}/CaM-dependent protein kinase (6), protein kinase activity was not detected in the purified BM-V used in the present studies.[2] Therefore, the Ca^{2+} regulation of BM-V is similar to the myosin-linked mechanisms observed for the Ca^{2+} regulation of molluscan myosin-II and vertebrate myosin-I. Regulation in molluscan myosin-II involves light chain mediated Ca^{2+} binding (38), which results in tight control over the phosphate release step of the ATPase cycle (39). In BB myosin-I, multiple copies of CaM serve as Ca^{2+}-binding light chains, which stimulate Mg-ATPase activity in the presence of Ca^{2+} (11). Although BM-V is similar to BB myosin-I in having multiple CaMs bound to the neck region, the actin-activated Mg-ATPase of BM-V is much more tightly regulated by Ca^{2+}. It is also important to note that in addition to the 4 CaM light chains, per heavy chain, BM-V also has 1 each of two distinct light chains of 17- and 23-kDa, which may also interact with the neck domain and participate in regulation (7).

The highly cooperative nature of the Ca^{2+} activation curve in the range of 1–3 μM (Fig. 2) is reminiscent of the Ca^{2+} activation curves of other CaM-regulated enzymes (40) and is consistent with known affinity constants for Ca^{2+} binding to CaM determined under ionic conditions similar to those used here (41). Thus our data support the hypothesis that Ca^{2+} is acting on BM-V via binding to its CaM light chains. Based on studies of BB myosin-I, two, not necessarily mutually exclusive, modes of CaM light chain regulation have been proposed (reviewed in Ref. 11). One is through Ca^{2+}-dependent dissociation of CaM from the heavy chain, and we report here that Ca^{2+} in the micromolar range does indeed result in partial (~30%) CaM light chain dissociation. The other mode is through allosteric regulation induced by Ca^{2+} binding to neck-bound CaM light chains.

A number of key questions remain. What is the nature and location of the putative non-CaM light chains? What role could Ca^{2+} binding to distal CaMs play in the regulation of ATP turnover in the head, if any? Is the structural basis for regulation via light chains basically the same for all myosins, no matter how large their neck domain?

Calpain Cleavage of the BM-V Tail—The Ca^{2+}-dependent protease calpain is known to cleave a number of proteins into stable domains, and in several cases cleavage by calpain has been suggested to play a physiological role (13). The localization of a calpain cleavage site one amino acid away from the PEST region is an intriguing finding since PEST regions have previously been hypothesized to be associated with cleavage by calpain (13). Our results support the idea that PEST regions form specific calpain recognition sites or form flexible interdo-

[2] F. Espindola, unpublished data.

TABLE II
Amino acid compositions of purified 65- and 80-kDa fragments of BM-V cleaved by calpain and comparison with molar ratios based on sequence data

Amino acid	65 kDa				80 kDa			
		Analysis[a]	Sequence[b]	Difference[c]		Analysis[a]	Sequence[b]	Difference[c]
	pmol	*mol/mol*	*mol/mol*	*%*	*pmol*	*mol/mol*	*mol/mol*	*%*
Asx	891	73	67	8.9	760	70	62	12.9
Glx	1060	86	74	16.2	1451	133	127	4.7
Ser	477	39	37	5.4	511	47	41	14.6
Gly	458	39	31	19.3	419	38	26	46.1
His	163	13	17	23.5	105	10	10	0
Arg	503	41	24	70.8	542	50	38	31.6
Thr	323	26	27	3.7	344	32	32	0
Ala	747	61	50	22.0	495	45	34	32.3
Pro	494	40	29	37.9	487	45	28	60.7
Tyr	284	23	31	25.8	143	13	13	0
Val	239	20	31	35.5	335	31	38	18.4
Met		15[d]	15	8		19[d]	19	0
Cys		12[d]	12	0		8[d]	8	0
Ile	314	26	44	40.9	278	26	41	36.6
Leu	623	51	57	10.5	914	84	93	9.7
Phe	265	22	27	18.5	183	17	17	0
Lys	530	43	51	15.7	524	48	60	20
Trp		6[d]	6	0		3[d]	3	0
		Σ analysis-sequence (%) 355				Σ analysis-sequence (%) 288		

[a] The molar ratio of amino acids to polypeptide was calculated from the pmol values determined by amino acid analysis of the polypeptide, as described in "Experimental Procedures," using a molecular weight value calculated from the known primary sequency by arbitrarily choosing a possible cleavage site. For example, the values expressed in the Table are based on: (*a*) for the polypeptide with an apparent molecular mass of 65 kDa, - a cleavage site 630 amino acids from the N-terminal, which gives a molecular weight of 71,793; and (*b*) for the polypeptide with an apparent molecular weight of 80 kDa, a cleavage site at Arg_{1140}, giving a molecular weight of 79,319 for the C-terminal polypeptide.

[b] Once the cleavage site is choosen, the exact molar ratio of amino acids is determined from the known sequence.

[c] The difference between the calculated molar ratio based on amino acid analysis and the exact molar ratio based on sequence is expressed as percentage of the exact molar ratio.

[d] Met, Cys and Trp were not determined by the amino acid analysis. Therefore, absolute values were introduced into the calculations based on the sequence data.

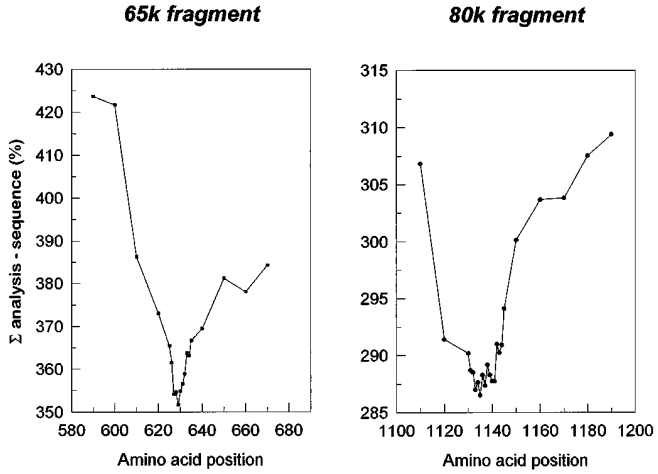

FIG. 6. **Prediction of the calpain cleavage site on BM-V that generates the 65-kDa polypeptide.** The sum of the differences between the calculated molar ratios based on amino acid analysis *versus* molar ratios based on the known sequence was determined as described in Table II for several chosen amino acid positions (Σ *analysis-sequence*) and plotted as a function of that position. The rationale was that the closer the fit between the molar ratio based on amino acid composition and the known sequence, the closer one would be to the cleavage site. This analysis shows a clear minimum, which points to a possible cleavage site near amino acid 629 ± 2 amino acids. To test this rationale, we performed the same procedure on the 80-kDa polypeptide, the exact cleavage site of which has been determined by microsequencing to be at Arg^{1140}. This analysis also showed a clear minimum (amino acid 1136 ± 5 amino acids) that included the known site. As an additional control, a 629-amino acid sequence from the C-terminal of BM-V was compared by this method to the 65-kDa fragment. The sum of the differences in this case was 614, a value well above the range of values obtained near the putative site.

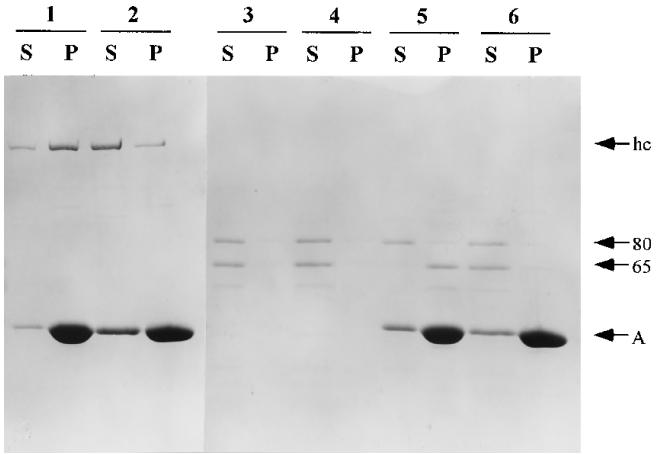

FIG. 7. **The 65-kDa head fragment, but not the 80-kDa tail fragment, binds to F-actin.** SDS-PAGE analysis of supernatant (*S*) and pellet (*P*) fractions of mock-digested BM-V (*lanes 1* and *2*) and BM-V calpain fragments cosedimented in the absence (*lanes 3* and *4*) or presence (*lanes 1, 2, 5,* and *6*) of 9 μM F-actin in the absence (*lanes 1, 3,* and *5*) and presence (*lanes 2, 4,* and *6*) of 2 mM ATP. Positions of actin (*A*), the intact BM-V heavy chain (*hc*), and the 80- and 65-kDa fragments are indicated.

main linkers that are especially susceptible to proteolytic cleavage. In the future, it will be important to determine if calpain cleavage of BM-V is a physiologically important process for severing the head from the tail domain.

Calpain Cleavage of BM-V Leads to a Loss of Ca^{2+} Regulation—Although the majority of actin-activated Mg-ATPase (65%) of BM-V is retained after limited proteolytic digestion by calpain, this ATPase is no longer regulated by Ca^{2+} (Fig. 8). The 65-kDa head domain resulting from calpain cleavage also cosediments with actin in an ATP-sensitive manner (Fig. 7). Thus, the abilities to hydrolyze ATP and to bind actin are retained in spite of calpain cleavage. We have used indirect methods to determine that the cleavage site producing the 65-kDa fragment is located near residue 629, at a position

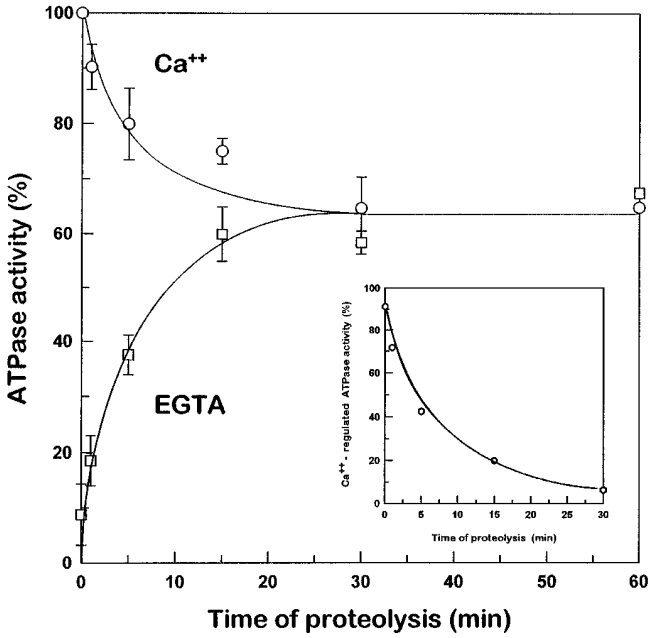

FIG. 8. **Increase in Ca^{2+}-independent, actin-activated ATPase activity accompanies the time course of calpain proteolysis of BM-V.** The actin-activated ATPase activity of BM-V was assayed after various times of proteolysis by calpain, both in the presence of 5 mM EGTA (□) or 0.5 mM free Ca^{2+} (○). The *inset* shows a plot of the Ca^{2+}-dependent, actin-activated ATPase activity over the time course of proteolysis.

equivalent to the proteolytically sensitive 50-kDa/20-kDa junction found in conventional myosins (reviewed in Ref. 42). On a gel filtration column, the 65-kDa fragment, together with CaM, elutes well before the volume expected for a globular 65-kDa protein.[3] These data suggest that, in spite of the cleavage, non-covalent domain interactions are maintained between the 65-kDa segment and at least a part of the neck domain with its CaM.

Other light chain-regulated myosins have suppressive neck domains that, in the absence of Ca^{2+}, are believed to prevent the interdomain rearrangements necessary for the ATPase cycle to occur. Experimental means of relieving the neck inhibition of regulated myosins include limited proteolytic digestion near the neck (10, 19). Biochemical removal of light chains also unregulates molluscan myosin (10) and smooth muscle myosin (42). Our data suggest that the neck domain of BM-V is similar to the other known regulated myosins in that it has a suppressive effect on the head domain. This effect can be relieved by proteolytic cleavage upstream of the head/neck junction. The major effect of calpain cleavage is thus to unregulate BM-V while leaving its enzymatic and actin binding properties relatively unimpaired.

Future Directions—A number of intriguing questions about the mechanochemistry of this unconventional myosin remain unanswered. A basic question is whether Ca^{2+} stimulates or inhibits BM-V motor activity in the cell. Even though Ca^{2+} greatly stimulates activity in Mg-ATPase assays, it leads to a reduction in the rate of actin filament movement in motility assays (7). Although the basis for this is behavior is unknown, there are many other examples where ATPase activity and motility assay behavior do not correlate well (43, 44). In the case of brain myosin-V, it is possible that absorption to nitrocellulose and other surfaces activates motor activity, perhaps due to interactions between the large neck domain of myosin-V and the surface (discussed in Ref. 11). Another question is why BM-V, which has a maximal ATPase similar to that of skeletal muscle myosin, moves more slowly in motility assays (~0.4 μm/s *versus* ~7 μm/s; Refs. 7 and 37). A final question is raised by the recent model that the neck domain of the conventional myosins, which consists of the two light chains wrapped around an extended α-helix (36, 38), might act as a lever to amplify a small conformational change in the head into a much larger power stroke of ~10 nm (reviewed in Ref. 32). This model suggests that BM-V, which has a neck domain three times larger than conventional myosins, should exhibit a step size that is also three times larger. On the other hand, extended necks (*i.e.* more than two IQ motifs) may have other functions besides ATPase regulation and mechanical transduction (for an example, see Ref. 45). Hopefully, the characterization and comparison of myosins from different classes will contribute to a better understanding of how the generic myosin motor works, as well as the specific cellular functions of each class.

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[3] S. Tauhata, unpublished data.

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