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

(Received for publication, September 26, 1995, and in revised form, May 1, 1996)

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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$^2+$ ($V_{\text{max}}$ 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 ($K_{\text{ATPase}}$ 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.

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 site(s) 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 or regions defined by coiled coils attached to a globular region of unknown function (7, 8). The hypothetical functions of the class V myosins and their novel 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 $K_{\text{ATPase}}$ 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.
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, cano (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 Ca2+. 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 ATPase activity. This domain did not bind to actin in cosedimentation assays and does not appear to contain a second actin-binding site.

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**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 0.5, 1.0, 5.0, and 10 μM, and 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.

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conditions that displace Mg²⁺ 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 (2.3 ATP/s; Ref. 11). These results indicate that BM-V has a modest ATPase of 1.8 ATP/s, depending on the preparation.

**TABLE I**

| Assay condition | ATPase activity (ATP/s per head) |
|-----------------|----------------------------------|
| K-EDTA          | Ca²⁺                             | Mg²⁺                             |
| 600 mM KCl     | 1.77 (±0.07)                     | 0.55 (±0.01)                     |
| 75 mM KCl      | 0.76 (±0.07)                     | 0.27 (±0.03)                     |

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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 mM CaM was included in all subsequent Mg-ATPase assays to guarantee maximal activity.

**FIG. 1.** The high Mg⁺⁺-ATPase activity of BM-V requires both Ca²⁺ 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 CaCl₂, 5.9 mM 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 Ca²⁺ exhibited some variation (0–3 ATP/s), as did the basal Mg-ATPase activity in the presence of Ca²⁺ (0–2 ATP/s).

**FIG. 2.** Ca²⁺ 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 mM F-actin, 5.9 mM additional CaM, and free Ca²⁺ 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. 3A.** 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
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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. 5A). 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. 5B), whereas the 80-kDa fragment was recognized only by tail antibodies (Fig. 5C). A diagram of the putative localization of these sites is shown in Fig. 5D.

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. 5E) and is located between Arg$^{1140}$ and Met$^{1141}$, just one amino acid downstream of 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. 5E). This same difference in a single amino acid appears in the two published primary sequences of chicken BM-V based on cDNA (Fig. 5F; 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 activity observed 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).

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. 3B) 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
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 possbility 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 \(\mu\)M purified skeletal muscle F-actin and 3 \(\mu\)M 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. 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 \(\mu\)M) pelleted with F-actin (0–10 \(\mu\)M) in the presence of either 4 mM EGTA (□) or 10 \(\mu\)M free Ca\(^{2+}\) (■). Bars, S.D.
Although we have previously shown that the tail domain of BM-V is a substrate for Ca\(^{2+}/\text{CaM}\)-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 \(\mu\)M (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 (\(\approx 30\%\)) 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 interdomain

\(^2\) F. Espindola, unpublished data.
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

### Table II

| Amino acid | 65 kDa pmol | 65 kDa mol/mol | 80 kDa pmol | 80 kDa mol/mol | Difference % |
|------------|-------------|----------------|-------------|----------------|--------------|
| Glx        | 1060        | 86             | 1451        | 133            | 127          | 4.7         |
| Ser        | 477         | 39             | 511         | 47             | 41           | 14.6        |
| Gly        | 458         | 39             | 419         | 38             | 26           | 46.1        |
| His        | 163         | 13             | 105         | 10             | 0            | 0           |
| Arg        | 503         | 41             | 542         | 50             | 38           | 31.6        |
| Thr        | 323         | 26             | 344         | 32             | 0            | 0           |
| Ala        | 747         | 61             | 495         | 45             | 34           | 32.3        |
| Pro        | 494         | 40             | 487         | 45             | 28           | 60.7        |
| Tyr        | 284         | 23             | 143         | 13             | 13           | 0           |
| Val        | 239         | 20             | 335         | 31             | 38           | 18.4        |
| Met        | 15          | 15             | 19          | 19             | 0            | 0           |
| Cys        | 12          | 12             | 8           | 8              | 0            | 0           |
| Ile        | 314         | 26             | 278         | 26             | 41           | 36.6        |
| Leu        | 623         | 51             | 914         | 84             | 93           | 9.7         |
| Phe        | 265         | 22             | 183         | 17             | 17           | 0           |
| Lys        | 530         | 43             | 524         | 48             | 60           | 20          |
| Trp        |             | 6              |             |                 |              |             |

### Analysis-sequence (%) 355

| Amino acid | 65 kDa pmol | 65 kDa mol/mol | 80 kDa pmol | 80 kDa mol/mol | Difference % |
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| Trp        |             | 6              |             |                 |              |             |

### Analysis-sequence (%) 288

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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.

Acknowledgments—We thank Domingus E. Pitta and Silvia Regina Andrade for technical assistance throughout this work. Special thanks to Claudia Rosa and Dr. Lewis J. Greene for the protein design and production, and Joseph V. Belsky Center for Protein Chemistry, Medical School of Ribeirão Preto, for the amino acid analysis and Dr. Lewis J. Greene for the idea to use the data to pinpoint the cleavage site.

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