Partial Deduced Sequence of the 110-kD–Calmodulin Complex of the Avian Intestinal Microvillus Shows that this Mechanoenzyme Is a Member of the Myosin I Family

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Abstract. The actin bundle within each microvillus of the intestinal brush border is laterally tethered to the membrane by bridges composed of the protein complex, 110-kD–calmodulin. Previous studies have shown that avian 110-kD–calmodulin shares many properties with myosins including mechanochemical activity. In the present study, a cDNA molecule encoding 1,000 amino acids of the 110-kD protein has been sequenced, providing direct evidence that this protein is a vertebrate homologue of the tail-less, single-headed myosin I first described in amoeboid cells. The primary structure of the 110-kD protein (or brush border myosin I heavy chain) consists of two domains, an amino-terminal "head" domain and a 35-kD carboxy-terminal "tail" domain. The head domain is homologous to the S1 domain of other known myosins, with highest homology observed between that of *Acanthamoeba* myosin IB and the S1 domain of the protein encoded by bovine myosin I heavy chain gene (MIHC; Hoshimaru, M., and S. Nakanishi. 1987. *J. Biol. Chem.* 262:14625–14632). The carboxy-terminal domain shows no significant homology with any other known myosins except that of the bovine MIHC. This demonstrates that the bovine MIHC gene most probably encodes the heavy chain of bovine brush border myosin I (BBMI). A bacterially expressed fusion protein encoded by the brush border 110-kD cDNA binds calmodulin. Proteolytic removal of the carboxy-terminal domain of the fusion protein results in loss of calmodulin binding activity, a result consistent with previous studies on the domain structure of the 110-kD protein. No hydrophobic sequence is present in the molecule indicating that chicken BBMI heavy chain is probably not an integral membrane protein. Northern blot analysis of various chicken tissue indicates that BBMI heavy chain is preferentially expressed in the intestine.

THE plasma membrane of most cells is underlaid by a complex array of cytoskeletal elements, the most common of which are actin filaments (for review of membrane-cytoskeleton interactions see Niggli and Burger, 1987). The apical brush border surface of the intestinal epithelial cell has proved to be a useful model system for investigating both the structural and molecular basis for actin–membrane interaction (reviewed in Mooseker, 1985; Louvard, 1989). Each microvillus of the brush border is supported by a uniformly polarized bundle of actin filaments. The barbed, preferred assembly ends of the filaments in this bundle are tightly opposed to the membrane at the microvillus tip. This bundle is also tethered laterally to the membrane by spirally arranged bridges (reviewed in Mooseker, 1985). These bridges are composed of a complex formerly termed 110-kD–calmodulin (for references see Coluccio and Bretscher, 1989; these workers have reconstituted the spiral bridges in vitro by addition of purified 110-kD–calmodulin to actin bundles). Studies from several laboratories have shown that 110-kD–calmodulin complex purified from chicken brush borders shares many structural and enzymatic properties with myosins (Collins and Borysenko, 1984; Howe and Mooseker, 1983; Mooseker et al., 1986; Conzelman and Mooseker, 1987; Coluccio and Bretscher, 1987, 1988; Krizek et al., 1987; Swanljung-Collins et al., 1987; Carboni et al., 1988) including mechanochemical activity (Mooseker and Coleman, 1989). Since these studies demonstrate that 110-kD–calmodulin is operationally a myosin, it has been termed brush border myosin I (BBMI[1]; Mooseker and Coleman, 1989) using the terminology suggested by Korn and Hammer (1988) for single-headed myosins (versus myosins II, for two-headed myosins). The 110-kD protein is referred in the text as heavy chain of the 110-kD–calmodulin complex.

1. *Abbreviations used in this paper*: BBMI, brush border myosin I; MIHC, myosin I heavy chain gene.

Studies on the functional properties of avian BBMI as well as the domain structure of its heavy chain indicate that the similarity of this protein to amoeboid myosin Is may extend beyond the lack of a long alpha helical tail. There is evidence that like BBMI, *Acanthamoeba* myosin I is associated with membranes (Matsudaira and Burgess, 1979; Adams and Pollard, 1986). Like *Acanthamoeba* myosin Is (reviewed in Korn and Hammer, 1988), BBMI, is an ATP-dependent crosslinker of actin filaments (Howe and Mooseker, 1983; Conzelman and Mooseker, 1987). It has not yet been demonstrated if BBMI contains an ATP-insensitive actin binding site comparable to that found on the carboxy-terminal end of myosin Is (Korn and Hammer, 1988). The heavy chain of BBMI consists of two discrete functional domains, and thus is analogous in structure to *Acanthamoeba* myosin I (Korn and Hammer, 1988). There is an 80–90-kD myosin head domain that contains the ATP binding site, the ATP-sensitive actin binding site, and a 20–30-kD domain at the presumed carboxy-terminal end of the molecule containing the calmodulin binding sites (Carboni et al., 1988; Coluccio and Bretscher, 1988). Although there is no direct evidence for the involvement of calmodulin in regulating the activity of this mechanoenzyme, calcium ion concentrations in the 1–10-μM range have significant effects on its activities. This includes an activation of its MgATPase activity both in the absence and presence of actin (Conzelman and Mooseker, 1987; Swanljung-Collins et al., 1987; Mooseker and Coleman, 1989), potentiation of actin binding under certain conditions (Coluccio and Bretscher, 1987), and activation of motility in vitro (Mooseker and Coleman, 1989).

Although the studies summarized above demonstrate that BBMI is a myosin-like mechanoenzyme, the relationship of this protein to *Acanthamoeba* myosin I or any other known myosin at the level of primary structure has not been determined. In the present study, we report results on the isolation and characterization of a cDNA molecule that encodes 1,000 amino acids (114,330 D) of the heavy chain of chicken BBMI. As expected from the functional and structural studies summarized above, sequence analysis indicates that the heavy chain of BBMI contains an amino-terminal domain that is homologous in sequence to the S1 domain of other known myosins, particularly that of *Acanthamoeba* myosin IB and the recently characterized myosin I heavy chain cDNA of the cow (Hoshimaru and Nakanishi, 1987). The carboxy-terminal 35-kD domain shows no sequence homology with conventional myosins or *Acanthamoeba* myosin IB but is homologous to the carboxy-terminal domain of the protein encoded by the bovine myosin I heavy chain gene. This indicates that the bovine gene most probably encodes the heavy chain of bovine BBMI as originally suggested by Hoshimaru and Nakanishi (1987), since this gene is preferentially expressed in intestinal tissue.

Materials and Methods

Bacterial Strains, Vectors

Escherichia coli strain Y1090 and lambda gt11 Eco RI–digested vector DNA were obtained from Amersham International Pic (Buckinghamshire, England). The *Escherichia coli* strain Y1089 was lysogenized with the lambda gt11 clone of interest as previously described (Snyder et al., 1987).

Total RNA Extraction and Northern Blot Analysis

Total RNA from chicken tissues homogenized in presence of guanidium isothiocyanate were isolated by precipitation with LiCl (Tronik et al., 1987). PolyA$^+$ RNA was obtained by passage of total RNA over oligo dT cellulose (Aviv and Leder, 1972).

Aliquots (10 μg) of total RNA samples were fractionated by electrophoresis on 1% agarose gel in the presence of 1 M formaldehyde (Lehrach et al., 1977), and transferred to nitrocellulose. Blots were prehybridized at 48°C for 16 h in 50% formamide, 4× SSC, 0.05 M Na_2HPO_4 pH 7.4, 1× Denhardt's solution, 250 μg/ml denatured salmon sperm DNA, and 500 μg/ml tRNA. Filters were hybridized with ^{32}P-labeled nick-translated DNA probe (20 ng/ml, sp act 1×10^9 cpm/μg) for 48 h at 48°C. Blots were then washed for 20 min twice in 2× SSC, 0.1% SDS at room temperature, and once in 0.1× SSC, 0.1% SDS at 65°C.

cDNA Library Construction and Immunoscreening

The cDNAs used for library construction were synthesized according to the method described by Gubler and Hoffman (1983) using 3 μg of polyA$^+$ RNA prepared from chicken intestinal mucosa. After methylation of the double strand cDNA with Eco RI methylase, Eco RI linkers were ligated to the blunt-ended cDNA, digested with Eco RI, and separated on an ultrogel column (type AcA 34; LKB Instruments, Gaithersburg, MD) in 20 mM Tris-HCl pH 7.5, 1 mM EDTA. 50 ng cDNA were then ligated to 1 μg of Eco RI–digested lambda gt11 vector DNA and ligated particles were packaged in vitro to generate a cDNA library containing 10^6 independent recombinants.

The lambda gt11 library was screened (for methods see Snyder et al., 1987) with a cocktail of two different polyclonal antisera raised against the heavy chain of BBMI (Shibayama et al., 1987) using alkaline phosphatase–conjugated secondary antibodies (Promega Biotec, Madison, WI). Positive plaques were subsequently screened with two monoclonal antibodies, CX-1 and CX-7, which most likely bind to epitopes at opposite ends of the molecule. CX-1 binds to an epitope within a 36-kD tryptic peptide from the presumed amino-terminal end of the heavy chain which contains the ATP binding site (Carboni et al., 1988); CX-7 most probably recognizes an epitope at the carboxy-terminal end of the molecule since it reacts with the intact molecule but not with a 90-kD fragment which like the parent molecule has a blocked amino terminus.

DNA Sequence Analysis

The dideoxy-chain termination procedure described by Sanger et al. (1977) was used to determine on both strands the overlapping sequence of cDNA restriction fragments subcloned into m13mp18-mp19 derivatives. The chemical procedure (Maxam and Gilbert, 1977) was applied to confirm the sequence in the 3' end fragment Sac I–Eco RI.

Amino Acid Sequence Analysis

Brush border myosin I used for microsequence analysis was purified from avian brush borders as described in Conzelman and Mooseker, 1986. 100 μg of BBMI were dissolved in gel sample buffer supplemented with 3% SDS. 5 μg of *Staphylococcus aureus* V8 protease were added and the mixture loaded on a gel. It was allowed to concentrate as a sharp band in the stacking gel before switching off the power supply. Gel in situ digestion was carried out for 20 min before restarting the electrophoresis. The generated fragments were then separated on a 20% gel and electroblotted onto poly(4-vinyl-*N*-methylpyridin)-coated glass fiber membranes. For details see Bauw et al., 1987. Immobilized protein bands were detected by dilute fluorescamine staining and excised for gas-phase sequencing. This was done with a sequenator (model 470 A; Applied Biosystems, Foster City, CA) equipped with an on-line 120 A phenylthiohydantoin–amino acid analyzer.

Analysis of Betagalactosidase-BBMI Heavy Chain Fusion Protein

Immunoblot Analysis. Expression of fusion protein was induced in lysogenized strains of *E. coli* Y1089 as described by Snyder et al. (1987). For immunoblot analysis of the fusion protein, bacteria (induced or noninduced controls) were collected by sedimentation at 10,000 *g*, washed in 10 mM Tris-HCl, pH 7.5 containing 0.2 mM PMSF. The cell pellets were solubilized in 10 vol SDS-PAGE sample buffer, subjected to SDS-PAGE (Matsudaira and Burgess, 1978), and electrotransferred to nitrocellulose by the method of Towbin et al. (1979). Immunoblot analysis was performed using the two polyclonal antisera described above and a battery of 30 monoclonal antibodies reactive with the heavy chain of BBMI.

Calmodulin Binding. To assay for calmodulin binding activity, SDS gels

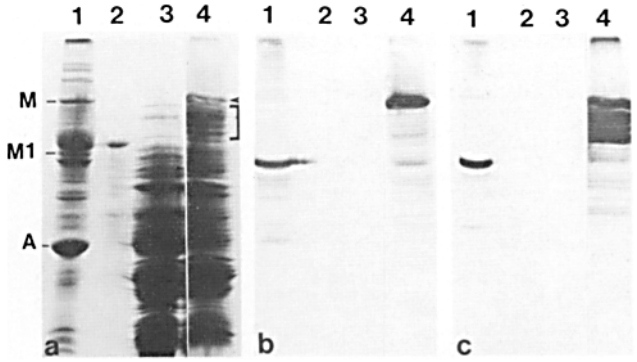


Figure 1. Immunoblot analysis of betagalactosidase fusion protein encoded by the N25 cDNA clone. (*a*) SDS-PAGE of isopropyl-β-D-thiogalactopyranoside (IPTG)-induced bacteria lysogenized with the N25 phage. Lane *1*, chicken brush border; lane *2*, betagalactosidase; lane *3*, uninduced bacteria; lane *4*, induced bacteria. The arrowhead indicates the position of the highest relative molecular mass fusion protein band. The bracket indicates the cascade of bands that are presumably proteolytic fragments of the fusion protein. The migration positions of the heavy chains for conventional brush border myosin (*M*) and brush border myosin I (*MI*) and actin (*A*) are indicated. (*b* and *c*) Immunoblot analysis of the gel in *a* with the monoclonal antibodies CX-7 (*b*) and CX-1 (*c*). These monoclonal antibodies are reactive with epitopes near the carboxy- and amino-terminal ends of the BBMI heavy chain.

containing lanes of induced cells were incubated with ^{125}I-calmodulin using methods described in Carlin et al. (1980).

Computer Analysis of the Amino Acid Sequences

Amino acid sequence comparisons were carried out according to the program described by Wilbur and Lipman (1983). The protein library from the National Biochemical Research Foundation (Washington, DC) was used for the search of homologous protein sequences.

Results

Isolation of a cDNA Clone Coding for the Heavy Chain of Chicken Brush Border Myosin I

The lambda gt11 library was first screened with a mixture of two polyclonal antisera raised against the heavy chain of chicken brush border myosin I. 30 positive clones were isolated and screened again with two monoclonal antibodies, CX-1 and CX-7, which are reactive with epitopes positioned at opposite ends of the heavy chain (see Materials and Methods). Among the 30 positive clones only one of these, N25, was recognized by both monoclonal antibodies and consequently this clone was selected for analysis. Before sequencing, the tentative identity of the clone as encoding the heavy chain of avian brush border myosin I was verified by assessing the immunoreactivity and calmodulin binding properties of a fusion protein expressed in bacteria lysogenized with the phage containing the N25 insert.

Immunoblot Analysis of Fusion Protein Encoded by N25 cDNA Expressed in Bacteria. The lysogenized bacteria obtained with the N25 phage expressed a fusion protein that was immunoreactive with an extensive battery of antibodies raised against the 110-kD protein (30 monoclonal antibodies and two different polyclonal antisera).

The fusion protein had an apparent relative molecular mass slightly larger than the heavy chain of conventional brush border myosin (200 kD; Fig. 1 *a*). The fusion protein was highly susceptible to proteolysis by endogenous bacterial proteases, resulting in the presence of numerous fragments which ranged in relative molecular mass from 180 to 120 kD. These proteolytic fragments were predominantly derived by cleavages from the carboxy-terminal end of the fusion protein since the carboxy-terminal–directed monoclonal antibodies, CX-7, reacted with the intact fusion protein but not with the lower relative molecular mass fragments (Fig. 1 *b*). Conversely, the NH_2-terminal directed monoclonal antibodies, CX-1, as well as the 110-kD polyclonal and anti–betagalactosidase antisera (Fig. 1 *c*), reacted with both intact fusion protein and the cascade of lower relative molecular mass fragments. Most of the monoclonal antibodies gave a staining pattern similar to either the CX-1 or CX-7 monoclonal antibodies. Several monoclonal antibodies whose epitopes have been mapped to tryptic fragments derived from the carboxy-terminal half of the 110-kD protein (Mooseker, M., and J. Carboni, unpublished observations) gave a staining pattern intermediate of that of CX-1 and CX-7 (results not shown). Taken together, these results provide strong evidence that the cDNA isolated does encode a considerable portion of the BBMI heavy chain.

Calmodulin Binding Properties of the Fusion Protein Encoded by N25 cDNA. Another indication that the cDNA clone, N25, encodes the BBMI heavy chain is that the fusion protein binds calmodulin, as assayed by a gel overlay technique (Fig. 2). Consistent with the suggested location of the calmodulin binding sites at the presumed carboxy-terminal domain of the molecule (Carboni et al., 1988; Coluccio and

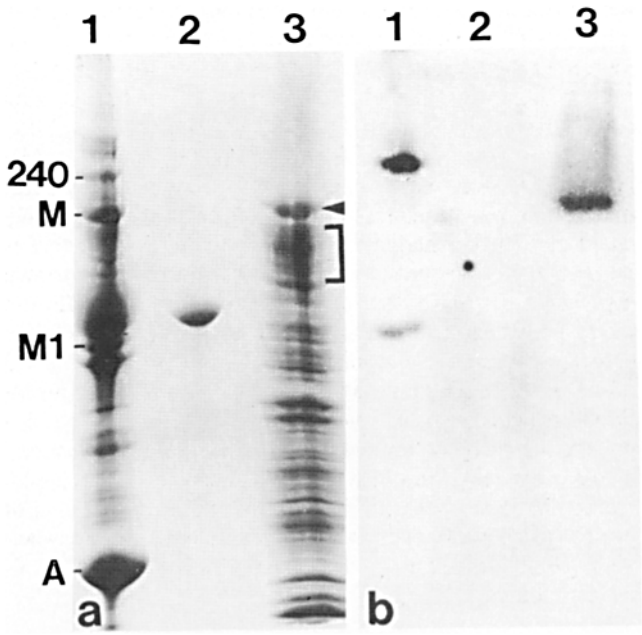
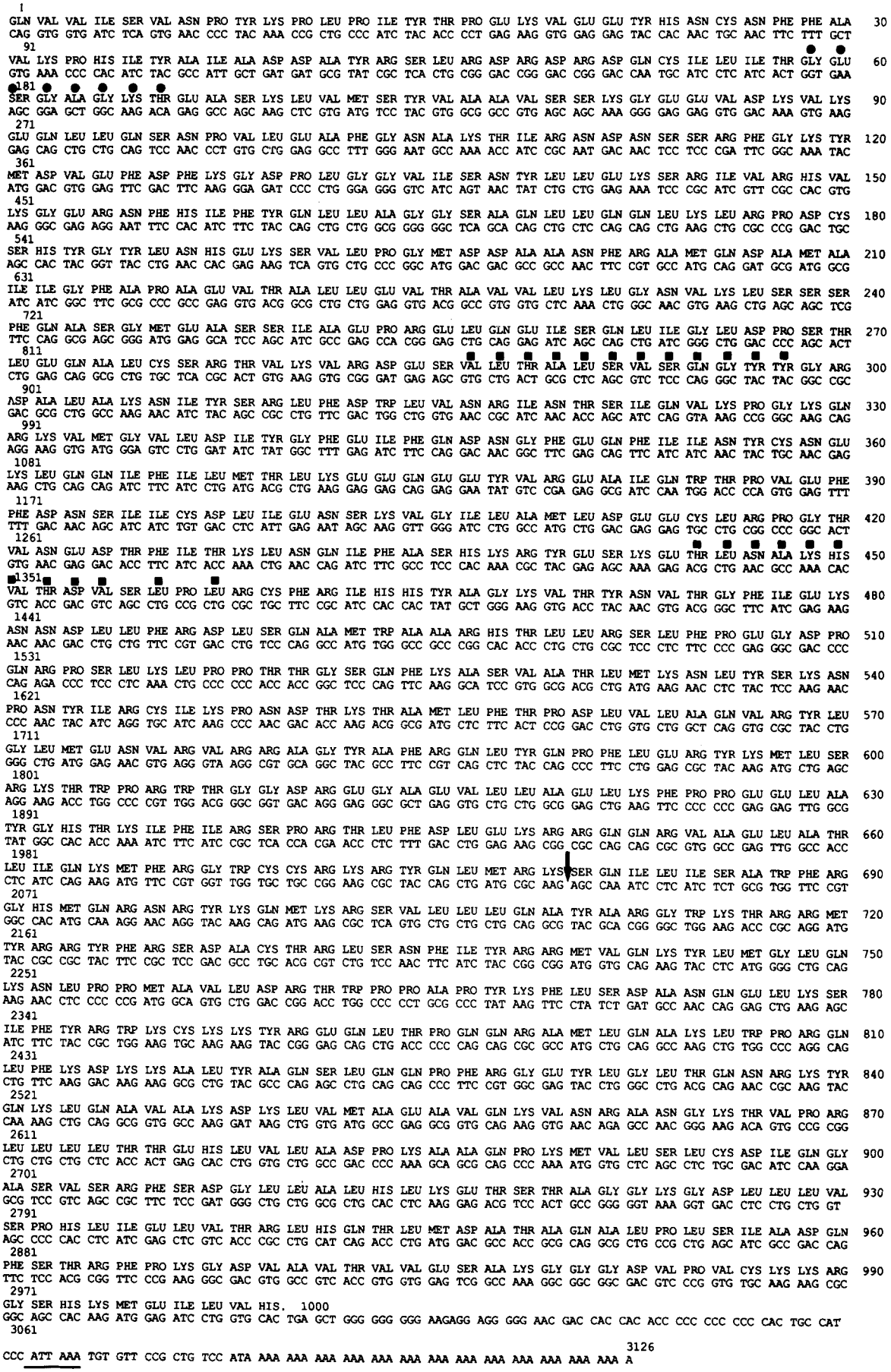


Figure 2. Analysis of calmodulin binding activity of the BBMI-betagalactosidase fusion protein. (*a*) Coomassie Blue–stained gel of (*1*) chicken brush borders, (*2*) betagalactosidase, and (*3*) IPTG-induced bacteria lysogenized with the N25 phage. Migration position of the ~200-kD fusion protein (*arrowhead*), its proteolytic fragments (*bracket*), and the alpha subunit of brush border spectrin (240 kD) are indicated. Other abbreviations are in Fig. 1. (*b*) Autoradiography of the gel in *a* after incubation with ^{125}I-calmodulin in presence of 1 mM $CaCl_2$. The 200-kD fusion protein but not the proteolytic fragments exhibits calmodulin binding, as do the alpha subunit of brush border spectrin and the BBMI heavy chain.



1
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AGC CCC CAC CTC ATC GAG CTC GTC ACC CGC CTG CAT CAG ACC CTG ATG GAC GCC ACC GCG CAG GCG CTG CCG CTG AGC ATC GCC GAC CAG
2881
PHE SER THR ARG PHE PRO LYS GLY ASP VAL ALA VAL THR VAL VAL GLU SER ALA LYS GLY GLY GLY ASP VAL PRO VAL CYS LYS LYS ARG 990
TTC TCC ACG CGG TTC CCG AAG GGC GAC GTG GCC GTC ACC GTG GTG GAG TCG GCC AAA GGC GGC GGC GAC GTC CCG GTG TGC AAG AAG CGC
2971
GLY SER HIS LYS MET GLU ILE LEU VAL HIS. 1000
GGC AGC CAC AAG ATG GAG ATC CTG GTG CAC TGA GCT GGG GGG GGG AAGAGG AGG GGG AAC GAC CAC CAC ACC CCC CCC CCC CAC TGC CAT
3061
3126
CCC ATT AAA TGT GTT CCG CTG TCC ATA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA A

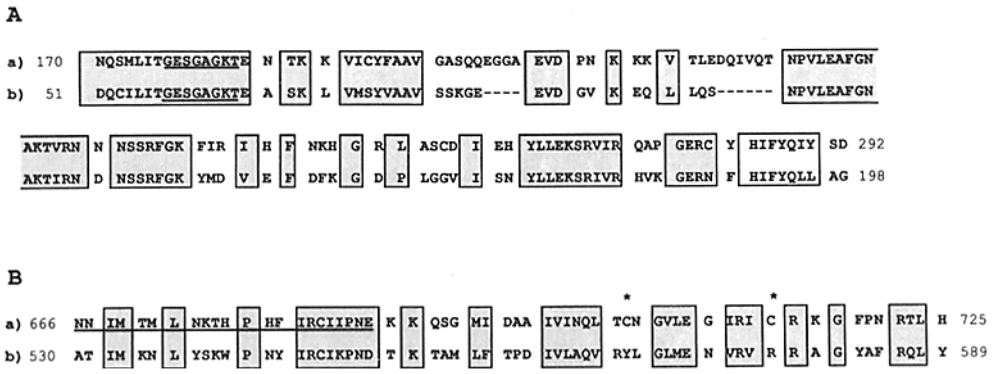

Figure 4. Comparison of ATP and actin binding regions of nematode myosin heavy chain and chicken BBMI. Amino acid sequence of the head portion of nematode myosin heavy chain (*a*) is aligned with that of chicken brush border myosin I heavy chain (*b*). (*A*) Region corresponding to the ATP binding site. The consensus sequence is underlined. (*B*) Region corresponding to the putative actin binding site. The putative amino acids involved in the actin binding site are underlined. The residues with SH groups are marked with asterisks. Identical and homologous amino acids are shown in lightly shaded boxes. The conservative replacements have been defined according to the following amino acid grouping: 1, R,K; 2, L,F,P,M,V,I; 3, S,T,Q,N,C; 4, A,G,W; 5, H; 6, E,D; 7, Y.

Bretscher, 1988), only the intact fusion protein exhibited calmodulin binding activity; the proteolytic fragments lacking the carboxy-terminal CX-7 epitope showed no detectable calmodulin binding activity by this assay even though these bands are present at concentrations comparable to that of the intact fusion protein (Fig. 2 *b*). As with the bona fide protein (Howe et al., 1982; Glenney and Glenney, 1985), optimal binding was observed in buffers containing Ca^{++}.

Nucleotide and Deduced Amino Acid Sequence of the N25 cDNA Clone. The 3,126-bp cDNA insert in the N25 clone was fully sequenced (Fig. 3). The 3′ end of this cDNA contains very short untranslated region (80 nucleotides) with an unusual polyadenylation signal (ATTAA) located 15 bp upstream of the polyA region (Wickens and Stephenson, 1984). The amino acid sequence deduced from the nucleotide sequence is shown in Fig. 3. There is an open reading frame of 3,000 nucleotides that encodes 1,000 amino acids with a calculated relative molecular mass of 114,330 D. Although this molecular mass is actually slightly larger than the apparent relative molecular mass of BBMI heavy chain, the absence of the ATG initiation codon and of the consensus sequence for initiation of translation in eukaryotic mRNAs (Kozak, 1986) indicates that the N25 clone is not full length. To further verify that the N25 clone encodes the heavy chain of BBMI, two V8 protease peptides of the heavy chain were sequenced; the protein fragments sequenced were 23, 15, and 10 kD, respectively. The two largest fragments showed the same NH_2-terminal sequence, corresponding with residues 445–458. The 10-kD band yielded a sequence matching residues 286–298 of the predicted sequence. These data prove that indeed the cDNA clone encodes 1,000 amino-acids of the avian BBMI heavy chain.

Chicken Brush Border Myosin I Heavy Chain Has a Structural Organization Similar to that of Myosins I

An Amino-terminal Domain Homologous to the Head Region of Conventional Myosins Heavy Chain. We compared the deduced amino acid sequence of the chicken BBMI heavy chain with that of the nematode skeletal muscle myosin heavy chain (Karn et al., 1983). The 680 amino-terminal residues of the BBMI heavy chain protein is homologous (32% of the amino acids are identical, 50% are homologous) to the S1 domain of the nematode myosin heavy chain. Furthermore, we found the consensus ATP binding sequence (Fig. 4 *A*) Gly-X-X-Gly-X-GLY-Lys-Thr (Higgins et al., 1986; for review see Warrick and Spudich, 1987), as well as a region containing the putative actin binding domain (Fig. 4 *B*). However, the two SH groups that affect ATPase activity in muscle myosin (but not in nonmuscle myosin), are replaced by nonconservative amino acids in the BBMI heavy chain (Lu et al., 1986). The homology between the two proteins is restricted to the S1 domain of myosin heavy chain. Indeed, no homology was found between the 35-kD carboxy-terminal domain of the brush border protein and the alpha helical tail domain of the conventional myosin heavy chain (McLachlan and Karn, 1982). Thus this analysis revealed that the heavy chain of BBMI consists of a myosin-like globular head domain (>80 kD) fused to a 35-kD carboxy-terminal domain.

Amino Acid Comparison with Myosin Is from Different Species. The tail-less myosin Is have first been described in *Acanthamoeba* (Pollard and Korn, 1973). In addition, Hoshimaru and Nakanishi (1987) have recently identified a bovine gene that they have termed myosin I heavy chain (MIHC) because it potentially encodes a 119-kD protein with an 85-kD amino-terminal domain that is homologous to the S1 domain of *Acanthamoeba* myosin IB. These authors have suggested that this gene encodes the bovine form of brush border myosin I since this gene is expressed in the intestine. A comparison of the chicken brush border myosin I sequence with that of bovine myosin I and *Acanthamoeba* myosin IB heavy chain indicates that these three proteins display strong homology within their amino-terminal S1 domains. The alignment is completely achieved by positioning the amino-terminal amino acid deduced from the N25 cDNA sequence with the amino acid in position 43 of the sequence deduced from the bovine MIHC cDNA and in position 42

Figure 3. Nucleotide sequence of BBMI heavy chain cDNA and its deduced amino acid sequence. The nucleotides are numbered in the 5′ to 3′ direction. The predicted amino acid sequence of BBMI heavy chain is shown above the nucleotide sequence. Amino acid residues marked with solid circles correspond to the consensus ATP binding sequence, residues marked with solid squares represent the sequences of the two BBMI heavy chain V8-generated peptides obtained by amino acid sequencing. The arrow indicates the end of the myosin globular head domain. The polyadenylation site is underlined.

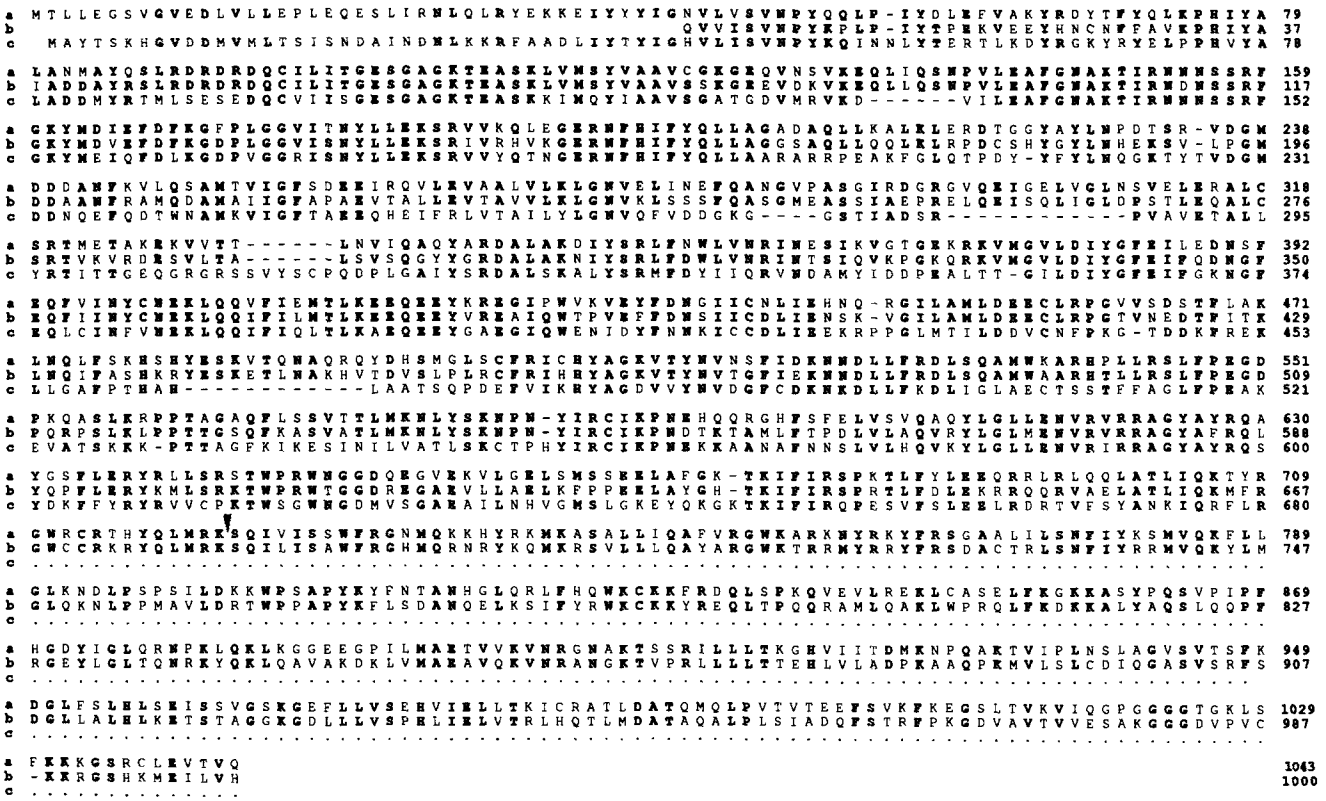

Figure 5. Sequence comparison of chicken brush border myosin I heavy chain and bovine and *Acanthamoeba* myosin Is heavy chains. Amino acid sequence of chicken brush border myosin I heavy chain (row *b*) was compared with that of bovine myosin I heavy chain (row *a*) (Hoshimaru and Nakanishi, 1987) and with the S1 domain of *Acanthamoeba* myosin IB (row *c*) (Jung et al., 1987). The amino acid sequence of the carboxy-terminal domain of *Acanthamoeba* myosin IB is not represented since no significant homology was found with the two other myosins. The identical amino acids are shown in bold characters. The end of the myosin globular head domain (reviewed in Warrick and Spudich, 1987) is indicated by an arrow.

of the *Acanthamoeba* myosin IB sequence (Fig. 5). Although in an individual pairwise comparison there is no gap between bovine and chicken myosin Is heavy chain in the S1 domain, the optimal alignment of both sequences with that of *Acanthamoeba* myosin IB heavy chain requires the introduction of five gaps of one to six residues. In addition this alignment with the amoeba sequence requires 12 gaps of 1 to 12 residues in this sequence. In this amino-terminal domain, 43% of amino acids are identical (60% are homologous) between brush border myosin I and *Acanthamoeba* myosin IB heavy chain, while 61% are identical (73% are homologous) between brush border myosin I and bovine myosin I heavy chain. Both the consensus ATP binding sequence and the putative actin binding domain are conserved between these three proteins (Fig. 5).

A comparison of the bovine sequence in the carboxy-terminal domain with that of the chicken brush border myosin I heavy chain indicates that these two proteins are also highly homologous in this region (48% of the amino acids are identical, 64% are homologous with a single gap of one residue). In striking contrast, no significant homology was found between these two proteins and the amoeba myosin I sequence within their respective carboxy-terminal domain. Since the homology covers the entire length of both chicken and bovine myosin Is (there is also significant homology at the nucleotide sequence level (60%), this suggests that these proteins display equivalent function in both species. Moreover these results are completely consistent with previous studies on the domain structure of the brush border myosin I heavy chain (Carboni et al., 1988; Coluccio and Bretscher, 1988), and confirms that this protein, like the *Acanthamoeba* myosin Is, lacks a conventional myosin tail.

Expression of Brush Border Myosin I Heavy Chain mRNA in Various Tissues

Previous studies using immunochemical or immunolocalization techniques have indicated that the heavy chain of BBMI is predominantly expressed in intestinal epithelia (Coudrier et al., 1981; Glenney et al., 1982; Shibayama et al., 1987). Northern blot analysis of total RNAs from small and large intestinal mucosa, as well as heart, gizzard, liver, and kidney, was carried out with a 940-bp DNA probe corresponding to the 3′ end restriction fragment Mlu I–Eco RI (Fig. 6 *B*). One band of 3.5 kb (comigrating with the large human villin mRNA; Arpin et al., 1988), consistent with the size of the protein, is detected in small and large intestine, but undetected in the other tissues (Fig. 6 *A*). These observations suggest that the mRNA encoding the heavy chain of BBMI is highly expressed in intestine and is not detectable by this method in the other tissues tested.

Discussion

In this paper we report the isolation of a cDNA clone that

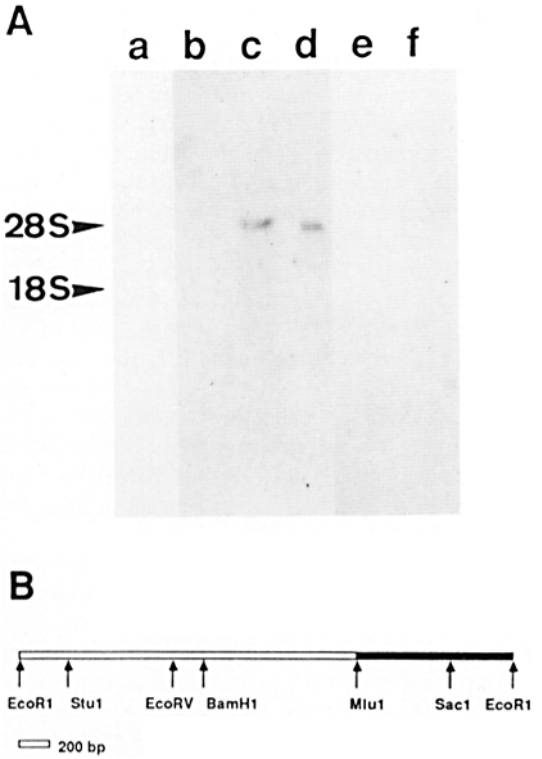

Figure 6. Hybridization analysis of total RNA from different chicken tissues. (*A*) 10 mg of total RNA extracted from gizzard (*a*), heart (*b*), small (*c*) and large (*d*) intestinal mucosa, liver (*e*), and kidney (*f*) were fractionated on 1% agarose/formaldehyde gel, transferred to nitrocellulose filter, and hybridized with ^{32}P-labeled DNA probe synthesized from the MLu I–Eco RI fragment (blackened section in *B*). (*B*) Restriction map of N25 cDNA clone showing unique restriction sites.

encodes 1,000 amino acids of the chicken BBMI heavy chain. Three lines of evidence verify the identification of this clone. First, the fusion protein encoded by this clone is immunoreactive with a battery of antibodies reactive with epitopes located throughout the BBMI heavy chain molecule. Second, the fusion protein expressed in bacteria binds calmodulin, and like the BBMI heavy chain, only the fusion protein with an intact carboxy terminus, as assayed by the presence of the CX-7 epitope, retains this binding activity. Third, and most convincingly, the amino acid sequences of two peptides, determined by protein sequencing methods, are both present within the deduced sequence. Taken together, these data verify that the N25 clone encodes the heavy chain of chicken BBMI.

Previous studies on BBMI purified from chicken intestinal epithelial cells have demonstrated that this complex is a mechanoenzyme that shares antigenic determinants with known myosins and also exhibits myosin-like functional properties (for references see Introduction). However, the possibility that BBMI is a novel type of actin-based motor, with little similarity to known myosins at the level of primary structure, was not excluded by these studies. The amino acid sequence deduced from the N25 cDNA clone encoding 1,000 amino acids of the chicken BBMI heavy chain now establishes that this mechanoenzyme is truly a myosin, based on the presence of an S1 domain homologous in sequence to all other known myosins (Figs. 4 and 5). Based on studies of the structural organization and sequence of known myosins (Fig. 7; for review see Warrick and Spudich, 1987; Korn and Hammer, 1988) two general classes of this protein with demonstrated mechanochemical activity have been characterized: conventional myosins (or myosin IIs) and the tail-less myosin Is first characterized in amoeboid cells but recently shown to be expressed in vertebrates as well (Hoshimaru and Nakanishi, 1987). Evidence for a third class of myosin-like proteins is provided by the recent characterization of the *nina* C gene in *Drosophila*. This gene encodes a pair of proteins from two overlapping mRNAs which are expressed in the rhabdom of the eye (Montell and Rubin, 1988). These proteins both consist of three domains: an amino-terminal kinase domain, a central myosin head domain most homologous in sequence to that of myosin Is, and a carboxy-terminal domain with no homology to the carboxy-terminal domains of other known myosins. The proteins encoded by the *nina* C gene have not been characterized with respect to their myosin-like properties. BBMI heavy chain and *nina* C proteins are structural proteins present specifically in microvillus cytoskeleton. It is possible to rescue *nina* C mutant flies that have shorter microvilli and shorter rhabdomeral diameter (Matsumoto et al., 1987) by introducing the normal *nina* C gene (Montel and Rubin, 1988). Thus, one may suggest that, like what is observed with *nina* C, BBMI could play an important role in morphogenesis and function of intestinal microvilli.

A comparison of the BBMI heavy chain sequence with that of other known myosins indicates that this protein is a member of the myosin I family. Moreover, the extensive sequence homology between the heavy chain of chicken BBMI and the deduced sequence for the protein encoded by the bovine MIHC gene provides strong support for the notion that the bovine gene encodes cow BBMI and not a distinct, as yet uncharacterized, isoform of myosin I. To achieve alignment of sequence between chicken BBMI and bovine MIHC, the first amino acid of the chicken protein sequence has to be posi-

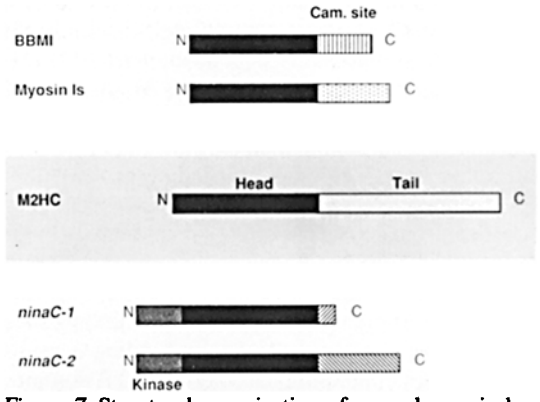

Figure 7. Structural organization of several myosin heavy chains. The members of the myosin I family, BBMI, myosin Is, *nina* C-1, and *nina* C-2, show an homologous head domain (S1) with the conventional myosin heavy chain (*M2HC*). Unlike the conventional myosins, the myosin Is display a shorter carboxy-terminal tail. No homology is found in this region between the different myosins. In the case of BBMI, calmodulin binding sites (*Cam. site*) have been assigned to this domain. The *nina* C proteins exhibit an additional amino-terminal domain with a putative serine/threonine kinase activity, joining the S1 domain.

tioned with amino acid 40 of the predicted protein encoded by the bovine gene (Fig. 5). Assuming that the extensive homology observed between these proteins continues through to the amino terminus, the N25 cDNA clone is lacking coding sequence for ~40 amino acids. However, because the N25 clone is incomplete, we cannot rigorously exclude the possibility that the BBMI heavy chain gene encodes a protein with a significantly larger amino-terminal domain, perhaps analogous to the *nina* C proteins of *Drosophila.* Two lines of evidence argue against this possibility. First, the molecular mass of the BBMI heavy chain, as estimated by SDS-PAGE (110 kD) is not consistent with the presence of a large amino-terminal domain. In addition the size of the mRNA for the BBMI heavy chain (3.5 kb) is appropriate for the relative molecular mass of this protein estimated by adding 40 amino acids to the relative molecular mass calculated from the N25 cDNA (~119 kD); this value is equivalent to the calculated relative molecular mass of the bovine MIHC protein (118,868 kD).

The sequence analysis presented here also provides insights into the functional properties of this new member of the myosin I family. This concerns key issues which have been raised previously by in vitro studies on BBMI including the molecular basis for the unusual calmodulin binding properties of the BBMI heavy chain, and the role the calmodulin subunits play in regulating the biological activity of this mechanoenzyme. There is also little known regarding how BBMI binds to the microvillar membrane. Insight into the first set of questions concerning the calmodulin association with 110-kD protein provided by its primary structure is largely confirmatory of previous studies. The overall domain structure of the BBMI heavy chain deduced from analysis of proteolytic fragments of the protein have suggested that heavy chain consists of a myosin head domain and a distinct calmodulin binding domain (Carboni et al., 1988; Coluccio and Bretscher, 1988). The primary structure of the heavy chain is consistent with this conclusion, and the calmodulin binding properties of the fusion protein (Fig. 2) support the notion that all the calmodulin binding sites reside within the carboxy-terminal 35-kD domain of the heavy chain. One still cannot exclude the possibility that the S1 domain contains calmodulin binding sites that are conformationally dependent upon the presence of an intact carboxy-terminal domain. An analysis of the amino acid sequence of the carboxy-terminal 35-kD domain has thus far proved uninformative, i.e., there is no homology with other known calmodulin binding proteins that have been sequenced. Recent studies show that secondary rather than primary structure is important for calmodulin binding. A putative alpha helix has been shown to be involved in calmodulin binding to *Bordetella pertussis* adenylate cyclase (Glaser et al., 1989). Mapping of the calmodulin binding sites may be facilitated by assessing calmodulin binding to well-defined fusion protein containing carboxy-terminal domain.

How BBMI interacts with the plasma membrane has been an issue of some controversy (for review see Mooseker, 1985). Based on the hydrophobic properties of the BBMI heavy chain, it has been suggested that this protein is an integral membrane protein (Glenney and Glenney, 1984). Other studies have indicated that BBMI is peripherally associated (for example, much of the BBMI is readily dissociated from isolated brush borders by addition of ATP) with the membrane. Moreover, porcine BBMI heavy chain is synthesized and assembled into the brush border surface with kinetics characteristic of a protein synthesized on free polysomes (Cowell and Danielson, 1985). Since it is highly probable that BBMI interacts with the microvillar core bundle via its S1 domain, membrane interaction most likely involves the opposite, carboxy-terminal end of the molecule. Hydropathy analysis (using the program devised by Kyte and Doolittle, 1982) of BBMI heavy chain sequence shows no significant hydrophobic region and strongly suggests the absence of a transmembrane domain (data not shown). Since the N25 clone is not full length, we cannot exclude the presence of a signal peptide/transmembrane domain at that end of the molecule. However this seems highly unlikely since such an orientation would position the myosin head domain at the bilayer. Moreover, such sequences are not present at the amino-terminal end of the coding region of the bovine BBMI heavy chain. Consequently it is likely that BBMI interacts directly or indirectly with an intrinsic membrane protein; for example the 140–200-kD glycoprotein shown to bind specifically to BBMI heavy chain immobilized on nitrocellulose (Coudrier et al., 1983). Alternatively, BBMI could interact directly with the bilayer either as a result of fatty acylation or through noncovalent interaction with phospholipids, as has been shown for a number of membrane skeletal proteins (Stadler et al., 1985; Burn and Burger, 1987; Staufenbiel and Lazarides, 1986; Keenan et al., 1982). It remains to study the physiological significance of the double interaction of BBMI with actin filaments (at the amino-terminal part of the heavy chain) and with membrane components (at the carboxy-terminal domain of the protein). The use of the cDNA encoding for BBMI heavy chain mutagenized in important regions (such as the ATP, actin, and calmodulin binding sites . . .), for transfection experiments could provide important clues to understand the biological functions of this protein.

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