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IDENTIFICATION OF BRUSH BORDER MYOSIN-I IN LIVER AND TESTIS

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Brush border myosin-I, or BBMI, constitutes the lateral links that connect in intestinal microvilli the core bundle of actin filaments to the membrane. Although related molecules have been identified in other higher eukaryotic tissues, northern blot analysis has indicated that the distribution of this particular myosin-I isoform is restricted essentially to intestine. Using reverse transcriptase polymerase chain reaction we have identified BBMI in a wide range of tissues including liver and testis. Our results also indicate that in testis the BBMI gene might be alternatively spliced.

Myosin-I is a class of protein identified by both biochemical and molecular biological techniques in a wide range of unicellular and multicellular eukaryotic organisms and thought to mediate translocation along microfilaments [1, 2, 3]; the various isoforms bear structural and biochemical resemblance to myosin-II, the mechanochemical molecule required for cytokinesis and muscular contraction [4, 5, 6].

In mammals, five different myosin-I genes have been identified to date: brush border myosin-I (BBMI) [7]; myosin-Iα (myr1) [8, 9]; myosin-Iβ (myr2) [9, 10, 11]; myosin-Iγ (myr4) [9, 12]; and myosin-IC (myr3) [13], which most closely resembles the myosin-I isoforms identified in protozoa. The NH_2-terminal region of the myosin-I isoforms is a globular domain containing both ATPase and actin-binding activities at separate sites [14, 15]. Just distal to this region is an α-helical calmodulin binding domain (the neck region). The COOH-terminal regions of the molecules

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are most divergent in sequence [16]; these regions may provide functional specificity by controlling subcellular localization.

BBMI was the first vertebrate myosin-I identified [17, 18, 19], and the first whose cDNA was cloned [7]. It is a major protein of the small intestinal brush border [20], and is visualized in electron micrographs as links between the microvillar F-actin bundle and the plasma membrane, appearing helically around the bundle at 33-nm intervals [20, 21, 22, 23]. The isolated BBMI is capable of supporting motility along microfilaments *in vitro* and is therefore a *bona fide* molecular motor [24, 25].

Northern blot analysis has suggested that expression of BBMI is restricted to intestine, stomach, and the trigeminal ganglion of the brain [7]. Using reverse transcriptase polymerase chain reaction (RT-PCR) we have found that rat BBMI mRNA is present at low levels in numerous tissues in which neither northern blot analysis nor immunoblotting can confirm its presence. Furthermore, we provide evidence that might indicate that in testis the BBMI gene is alternatively spliced.

Materials and Methods

Cloning and sequencing of myosin-I

Poly(A)$^+$ RNA was isolated from bovine and rat tissues using the mRNA Fast Track Kit (Invitrogen, San Diego, CA), according to the manufacturer's protocols. Two micrograms of poly(A)$^+$ RNA was put into each 20 µl reverse transcriptase reaction, using AMV reverse transcriptase (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. The primers used in these reactions were either $dT_{(12\text{-}18)}$ (Boehringer Mannheim) or the G or N1 primers described below. Prior to polymerase chain reaction (PCR), each reaction was stopped by adding 190 µl TE (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). PCR was carried out in 100 µl reactions with 5 µl first-strand cDNA (as obtained above) under standard conditions. The primers, derived from sequences of both the bovine BBMI cDNA and cDNA amplified from rat, are listed in Table I. PCR products were directly subcloned into either pT7Blue (Novagen, Madison, WI) or pCRII vectors (Invitrogen) using T4 DNA ligase (Gibco BRL, Gaithersburg, MD or Invitrogen) according to the manufacturers' protocols. Dideoxynucleotide chain-termination DNA sequencing was performed using either the Bst Premixed Standard Sequencing kit (Bio-Rad, Hercules, CA) or the Sequenase 2.0 kit (Amersham, Arlington Heights, IL). Sequencing reactions were run on 5% HydroLink Long Ranger (AT Biochem, Malvern, PA) gels. Sequence analysis was done using Genetics Computer Group computer software.

Anti-peptide antibody production

The peptide CPASGVCDGKGIQEIGEMMGLNS corresponding to amino acids 289 - 310 deduced from the rat liver cDNA sequence was synthesized, coupled using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) to keyhole limpet hemocyanin, separated from uncoupled

reagent, and used as antigen to make polyclonal antisera in rabbits. The serum was collected and affinity purified on BBMI isolated from rat intestine [18] and immobilized on nitrocellulose.

Immunoblotting

Proteins were separated on SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose. The nitrocellulose was incubated in 3% BSA, 10 mM Tris, pH 8.0, 150 mM NaCl for three hrs before addition of antibody at a 1:10,000 dilution. The nitrocellulose was washed with Tris-NaCl buffer before incubation with goat anti-rabbit IgG-alkaline phosphatase (Promega Corp., Madison, WI) at a 1:7500 dilution. Color development was with nitroblue tetrazolium (NBT) and bromo-4-chlor-3-indoyl phosphate (BCIP) per the manufacturer's instructions (Promega).

Results and Discussion

The first pair of non-degenerate oligonucleotide primers, whose sequences were derived from the bovine BBMI cDNA sequence, was initially used in RT-PCR of bovine liver mRNA (see Table I). A single band of 1.1 kb, the expected size for this segment of the BBMI gene, was amplified. Cloning and sequencing confirmed that this PCR product was identical to the appropriate segment of the BBMI cDNA from intestine, with the exception that the codon for cysteine 459 was missing. The results of this experiment indicated that in contrast to the previous report [7], mRNA encoding BBMI is present in *bovine* liver.

This same set of primers was used in RT-PCR with *rat* liver mRNA. A single band of 1.1 kb was amplified, cloned, and sequenced (see Fig. 2). Since the degree of sequence similarity to bovine BBMI is considerably

Table I. Oligonucleotide primers used. Oligonucleotide primers K and G were used in RT-PCR of mRNA of bovine liver and rat tissues to generate a fragment spanning nucleotides 329 to 1431 (numbered according to bovine BBMI; [7]). Primer N1 was derived from internal sequence of the K-G fragment amplified from rat liver and was used in conjunction with primer K to generate a fragment spanning nucleotides 329 to 645. Primer N2 was derived from the start of translation of bovine BBMI and was used with N1 to generate a fragment spanning nucleotides 1 to 645. Primer M1 was derived from the K-G fragment of rat and used with M2, derived from bovine sequence, to amplify the fragment from nucleotides 1320 to 2033 in rat liver and testis. Primer M3 was derived from the sequence of the M1-M2 fragment and used with M5, derived from bovine sequence, to amplify the fragment from nucleotides 1966 to 2575.

Upstream	Downstream
K 5'-GGCTAGTAAGCTAGTGATGTC-3'	G 5'-GGAGAAGAGCTGGTTCAGCTTTG-3'
N2 5'-ATGACCCTTCTGGAAGGTTCCG-3'	N1 5'-GGCCTTCAGCAGCTGTGTGTCTGC-3'
M1 5'-CCTCATTGAACATAGTCAGCGAGG-3'	M2 5'-ATCTTTGTCTTCCCAAAGGCCAGC-3'
M3 5'-CTGGAATGGGGAAGACCGGGAAG-3'	M5 5'-CCTTTTTGCCCTTGAACAGCTCGC-3'

higher than that of any currently known pair of myosin-I isoforms, we believe that the rat cDNA we identified is the orthologue of bovine BBMI, and we refer to the gene as rat BBMI. In addition, the codon corresponding to bovine BBMI cysteine 459 was present. Therefore, this pair of primers successfully amplified the appropriate segment of the BBMI cDNA from rat tissue, and the gene is expressed in rat liver.

We next attempted to determine whether rat BBMI cDNA was present in other tissues using RT-PCR with these same primers. A single 1.1-kb product was amplified from brain, lung, and kidney mRNAs. In testis, in addition to the 1.1-kb product we found a second major amplification product migrating at 1.3 kb. To examine this further, RT-PCR was performed on rat testis mRNA using a nested downstream primer derived from the sequence of the product amplified from rat liver (see Table I). There were three major products from this reaction. The smallest exhibited migration consistent with the expected size of 318 bp; sequencing confirmed its identity as the appropriate BBMI fragment. This fragment was found in the tissues examined above, as expected, and was also amplified from small intestinal mucosa, spleen, and heart. Another of the PCR products, when cloned and sequenced, was found to be 493 bp in size. Its sequence was identical to that of the original product but included a 175-bp insertion. The third product, approximately 530 bp, when cloned and sequenced, was found not to be homologous to myosin-I or to any other gene in the database, nor did it contain an open reading frame of significant length. These same results were obtained using testes from different rats. This set of experiments indicates that the BBMI gene is transcribed at least at low levels in numerous rat tissues, with a hitherto unknown variant present in testis.

The sequence of the insert contained in the testis-specific 493-bp product was analyzed (Fig. 1). It encodes a unique amino acid sequence not closely related to any sequences in the database, followed by a premature termination codon. The novel sequence begins after the asparagine residue corresponding to position 532 in the bovine BBMI gene and extends for 29 amino acids before reaching the stop codon. The 175-bp insertion in which this information is contained has consensus donor and acceptor sequences for splicing of a type II intron [26]. In addition, the position of this insertion is identical to the position of intron 7 of the bovine BBMI gene. Finally, the product resulting from PCR of rat genomic DNA using the primers encompassing this region of the cDNA was cloned and sequenced. This segment contained the entire testis-specific sequence in the appropriate location, and was large enough to contain other exons, as the intron-exon map of the bovine BBMI gene suggests it would [27]. Taken together, these data suggest that the 175-

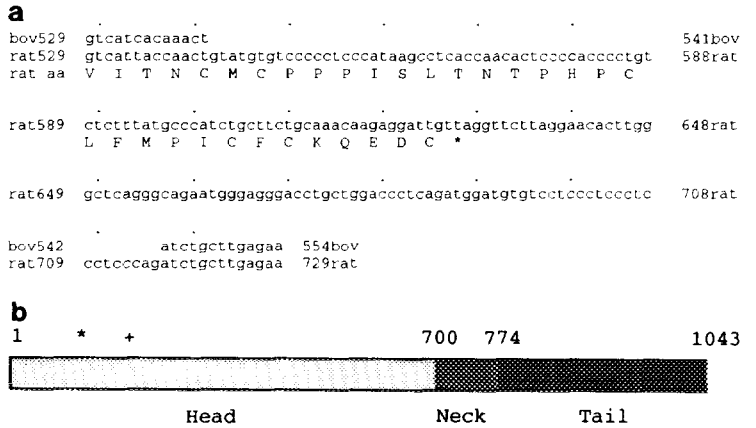

Figure 1. a) Nucleotide and deduced amino acid sequence of the region including the testis-specific splice variation. The top line corresponds to the published sequence of the bovine BBMI cDNA [7]. The second line is the sequence of the testis-specific PCR product (see text). The insert begins and ends where the bovine sequence stops and restarts, respectively. The third line is the deduced amino acid sequence of the putative translation product in the rat. b) Diagram of BBMI primary structure. Numbers represent amino acid residues at approximate boundaries between subdomains for bovine BBMI. *, P-loop; +, site of testis-specific splice variation.

bp insertion results from testis-specific aberrant or alternative splicing. Because of the premature termination codon, the protein encoded by this cDNA would contain the NH_2-terminal segment of the head including the ATP-binding site, followed by a short stretch of unique sequence (Fig. 1a). This molecule would contain neither the actin-binding region of the myosin-I head, the calmodulin-binding domains, nor the tail (Fig. 1b). The function of such a molecule, if it is expressed, can only be the subject of speculation.

To examine the possibility that other sites of alternative splicing exist in the rat testis BBMI gene, we attempted to amplify other segments of the cDNA using RT-PCR. A PCR "walk" to the 5' end of the coding region was performed using a non-degenerate upstream primer derived from the beginning of the coding region of the bovine BBMI cDNA (see Table I). At least two products were expected in testis, one representing the "traditional" cDNA and the other representing the splice variant. This was the case; the lower-molecular weight product included the 318-bp fragment as above, with the extra sequence corresponding to the 5' end of bovine BBMI. The higher-molecular weight band was approximately 175 bp larger in size, in accordance with the size of the insert we anticipated. There was no other amplified product.

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 26 tgggggtggaggatctcatcctcctggagcctctggatgaggagtctctgatcaagaacctccaacttcgttatgagaaaaaagagattt  115
  9   G  V  E  D  L  I  L  L  E  P  L  D  E  E  S  L  I  K  N  L  Q  L  R  Y  E  K  K  E  I  Y   38
116 atacgtacattgggaatgtggtgatctccatgaaccctaccagcagcttcctatctatggcccagagtttattgccaaatacgggact  205
 39   T  Y  I  G  N  V  V  I  S  M  N  P  Y  Q  Q  L  P  I  Y  G  P  E  F  I  A  K  Y  R  D  Y   68
206 ataccttctatgagctgaagccccacatctatgctttggcaaacgtggcctaccagtcgctgaaggatcgggaccgggaccagtgtatcc  295
 69   T  F  Y  E  L  K  P  H  I  Y  A  L  A  N  V  A  Y  Q  S  L  K  D  R  D  R  D  Q  C  I  L   98
296 tgatcactggcgagagtggagctggcaagactgaggctagtaagctagtgatgtcctatgtggctgctgtctgtgggaaaggggagcagg  385
 99   I  T  G  E  S  G  A  G  K  T  E  A  S  K  L  V  M  S  Y  V  A  A  V  C  G  K  G  E  Q  V  128
386 tgaactcggtgaaggagcagctgcttcagtcaaacccagtgctggaagcatttggcaatgccaagaccatccgcaatgacaactcctctc  475
129   N  S  V  K  E  Q  L  L  Q  S  N  P  V  L  E  A  F  G  N  A  K  T  I  R  N  D  N  S  S  R  158
476 gatttggaaaatacatggacatcgagtttgacttcaaagggtccccccttggaggtgtcattaccaactatctgcttgagaagtccagag  565
159   F  G  K  Y  M  D  I  E  F  D  F  K  G  S  P  L  G  G  V  I  T  N  Y  L  L  E  K  S  R  V  188
566 tggtgaagcaactcaaaggggaaaggaacttccatatcttctatcagctgctggctggagcagacacacagctgctgaaggccctaaagc  655
189   V  K  Q  L  K  G  E  R  N  F  H  I  F  Y  Q  L  L  A  G  A  D  T  Q  L  L  K  A  L  K  L  218
656 ttgaggaagatgcacgtggctatgcctaccttaatggggaggtgtccagggtgaacggtatggatgatgcctccaacttcagggctgtgc  745
219   E  E  D  A  R  G  Y  A  Y  L  N  G  E  V  S  R  V  N  G  M  D  D  A  S  N  F  R  A  V  Q  248
746 agaacgcaatggcagtgattgggttctcagaggaggagattagacaagtgctggaggtgacagccctggtactgaagctgggcaatgtga  835
249   N  A  M  A  V  I  G  F  S  E  E  E  I  R  Q  V  L  E  V  T  A  L  V  L  K  L  G  N  V  K  278
836 agctggcaggtgaattccaggccaacgggcttccagccagtggtgtctgtgatgggaaaggtattcaggagattggggagatgatgggtt  925
279   L  A  G  E  F  Q  A  N  G  L  P  A  S  G  V  C  D  G  K  G  I  Q  E  I  G  E  M  M  G  L  308
926 tgaactctgtagaactagagaaagctttgtcctcaagaaccatggagacaggcaaagagaaggtggtcacggtgctgaacgtcatccagg 1015
309   N  S  V  E  L  E  K  A  L  S  S  R  T  M  E  T  G  K  E  K  V  V  T  V  L  N  V  I  Q  A  338
1016 ctcagtatgctcgggatgccctggccaagaatatttacagccgactcttcgactggatagtgaatcgcatcaacgagagcatcaaggtgg 1105
339   Q  Y  A  R  D  A  L  A  K  N  I  Y  S  R  L  F  D  W  I  V  N  R  I  N  E  S  I  K  V  G  368
1106 gcactgggagaagaggaaggttatgggggtcctggatatctacggctttgagatactggaggataacagctttgagcagttcgtgatca 1195
369   T  G  E  K  R  K  V  M  G  V  L  D  I  Y  G  F  E  I  L  E  D  N  S  F  E  Q  F  V  I  N  398
1196 actactgcaatgaaaaactacagcaggtgtttatagaactgaccttgaaggaagaacaggaggagtataagagagaaggcataccatgga 1285
399   Y  C  N  E  K  L  Q  Q  V  F  I  E  L  T  L  K  E  E  Q  E  E  Y  K  R  E  G  I  P  W  T  428
1286 caaaggtggagtactttgacaacggcatcatctgtgacctcattgaacatagtcagcgaggcatcctggccatgctggatgaggagtgcc 1375
429   K  V  E  Y  F  D  N  G  I  I  C  D  L  I  E  H  S  Q  R  G  I  L  A  M  L  D  E  E  C  L  458
1376 tgagacctggggtggtcagtgacaccactttctagcaaagttgaaccagctttctccaagcacagtcactacgagagtaaagtcaccc 1465
459   R  P  G  V  V  S  D  T  T  F  L  A  K  L  N  Q  L  F  S  K  H  S  H  Y  E  S  K  V  T  Q  488
1466 agaatgctcagcgccaatacgaccgcaccatgggcctcagctgcttccgcatctgccactatgcgggcaaggtgacatacgacgtgacca 1555
489   N  A  Q  R  Q  Y  D  R  T  M  G  L  S  C  F  R  I  C  H  Y  A  G  K  V  T  Y  D  V  T  S  518
1556 gtttcatcgacaagaataacgacctactcttccgagacctatcccagacaatgtggaaggcccagcatccctcctccactccctgtttc 1645
519   F  I  D  K  N  N  D  L  L  F  R  D  L  S  Q  T  M  W  K  A  Q  H  P  L  L  H  S  L  F  P  548
1646 ccagaggtaatcccaaggaagcatctcccaaacggccccgactgctgggacccagtttaagaattcggtggccatactcatgaagaacc 1735
549   R  G  N  P  K  E  A  S  P  K  R  P  P  T  A  G  T  Q  F  K  N  S  V  A  I  L  M  K  N  L  578
1736 tgtattctaagaacccgaactacatcaggtgcataaagcccaatgaccagcagcagcaaggcaggttcacctcagaactggttatggtcc 1825
579   Y  S  K  N  P  N  Y  I  R  C  I  K  P  N  D  Q  Q  Q  Q  G  R  F  T  S  E  L  V  M  V  Q  608
1826 aggctcgatacctggggctgctggagaatgttcgggtgcgcagggctggctacgccttccgccaggcctataagcctttcctggagaggt 1915
609   A  R  Y  L  G  L  L  E  N  V  R  V  R  R  A  G  Y  A  F  R  Q  A  Y  K  P  F  L  E  R  Y  638
1916 accgattgctgagccggagtacgtggccgcgctggaatggggaagaccgggaaggtgtggagaaggtcctggggtctctgattttgtcat 2005
639   R  L  L  S  R  S  T  W  P  R  W  N  G  E  D  R  E  G  V  E  K  V  L  G  S  L  I  L  S  S  668
2006 cagaggagctggcctatgggaggacaaagatcttcatcagaagccccaagactcttttctacctggaagaacaaaggcgcctgaggcttc 2095
669   E  E  L  A  Y  G  R  T  K  I  F  I  R  S  P  K  T  L  F  Y  L  E  E  Q  R  R  L  R  L  Q  698
2096 agcagctggccaccctcatccagaaggtttaccggggctggcgttgtcggacccactaccagcagatgcggaagagtcagatcctgctct 2185
699   Q  L  A  T  L  I  Q  K  V  Y  R  G  W  R  C  R  T  H  Y  Q  Q  M  R  K  S  Q  I  L  L  S  728
2186 cggcttggtttcggggcaacaagcaaaagaagcactatgggaagatccggtcatcagtactgctgatccaggctttcgtgaggggctgga 2275
729   A  W  F  R  G  N  K  Q  K  K  H  Y  G  K  I  R  S  S  V  L  L  I  Q  A  F  V  R  G  W  K  758
2276 aggcccgcaagaactatcgaaaatacttccggtcaggcgcccgcatcactttggcaaattttatctaccagagcataaccagaaattcc 2365
759   A  R  K  N  Y  R  K  Y  F  R  S  G  A  R  I  T  L  A  N  F  I  Y  Q  S  I  T  Q  K  F  L  788
2366 tcttgaatctgaagaagaatttgccatccaccaaagtgttggacaacacgtggccagctgcccttacaggtgcttcaacacagccaatc 2455
789   L  N  L  K  K  N  L  P  S  T  K  V  L  D  N  T  W  P  A  A  P  Y  R  C  F  N  T  A  N  Q  818
2456 aggaactgcagcacctcttctaccagtggaagtgcaagaagtaccgagatcagctgtccccgaagcaggtgcagacactgagggaaaagc 2545
819   E  L  Q  H  L  F  Y  Q  W  K  C  K  K  Y  R  D  Q  L  S  P  K  Q  V  Q  T  L  R  E  K  L  848
2546 tctgtgcca                                                                                   2554
849   C  A                                                                                       851
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Figure 2. The partial nucleotide sequence and deduced amino acid sequence of rat BBMI heavy chain cDNA from overlapping RT-PCR fragments from rat liver and testis mRNA. Numbering is according to bovine BBMI heavy chain [7]. The two underlined nucleotides flank the site of the testis-specific putative alternate splice (see Fig. 1). Anti-peptide antibodies were prepared against the amino acid sequence underlined.

We proceeded toward the 3' end of the coding region in similar fashion, using downstream primers derived from the bovine orthologue at regions expected to be conserved based on homology with chicken BBMI. In all cases, there were no further splicing variants. Although we were successful in amplifying overlapping segments spanning most of the

coding region, the most distal segment of the rat BBMI gene was unable to be amplified. Presumably, this was because the very last portion of the coding region is poorly conserved with respect to bovine BBMI. The segment that was sequenced includes the head, all three putative calmodulin-binding domains, and the proximal 28% of the tail, a total of 81% of the coding region (Fig. 2). Over the region of the cDNA obtained by this method, the deduced amino acid sequence of the rat protein is 86% identical and 95% homologous to that of bovine BBMI (Fig. 3).

Using the deduced amino acid sequence, a peptide segment unique to rat BBMI was identified which, based on the structure of the S1 fragment of myosin-II, is expected to be located on the surface of the protein (see Fig. 3). Antibodies raised against this peptide were used in immunoblots and found to be specific for purified intestinal brush border myosin-I and not the myr1, myr 2, or myr 4 gene products which in liver are referred to as the 130-kDa, 110-kDa, and 105-kDa myosin-I's,

Figure 3. Comparison of the deduced amino acid sequence of the newly cloned rat BBMI segment (top) with that of the corresponding region of bovine BBMI (bottom). The sequences are 86% identical and 95% homologous (homology defined by A=G, S, T; C=S; D=E=N=Q; F=I, L, M, V, Y; H=K, N, Q, R; I=L=M=V; K=H=R; P=L; S=A, T; T=A, S, V).

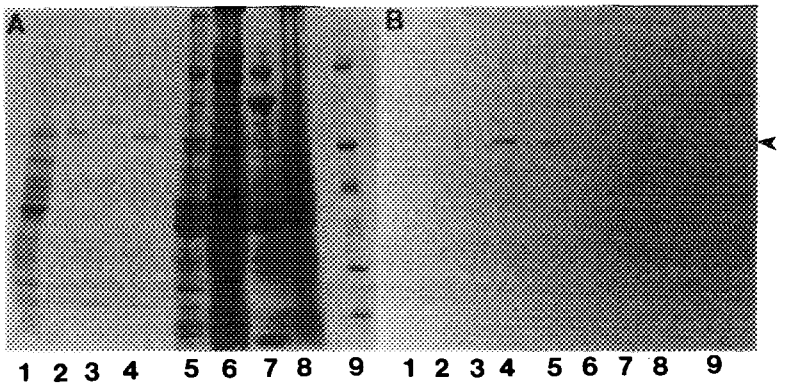

Figure 4. Immunoblot [Panel B] of extracts from intestine (lane 5), kidney (lane 6), liver (lane 7), and testis (lane 8) with affinity-purified anti-peptide antibodies. Lane 1, fraction enriched for rat liver 105-kDa myosin-I; lane 2, purified rat liver 110-kDa myosin-I; lane 3, purified rat liver 130-kDa myosin-I; lane 4, purified rat intestinal BBMI. The corresponding gel stained with Coomassie blue is in Panel A. Molecular weight markers in lane 9 include myosin, 200 kDa; phosphorylase B, 92 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; and soybean trypsin markers, 21 kDa. Arrow indicates position of BBMI.

respectively [28, 29]. Although a polypeptide of 110-kDa is identified in intestinal extracts by immunoblotting, no polypeptide of the expected molecular weight could be identified in extracts made in the presence of ATP [28] from liver, kidney or testis (Fig. 4). Although it is possible that no translation occurs in these other tissues, a more likely possibility is that the amount of BBMI protein in these tissues is substantially lower than that found in intestine.

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