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A second isoform of chicken brush border myosin I contains a 29-residue inserted sequence that binds calmodulin

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Chicken brush border myosin I (CBB-MI) is a single-headed, nonfilamentous, myosin-like mechanoenzyme which, as isolated, has 3 mol of calmodulin (CAM) 'light chains' bound per mole of 119 kDa heavy chain. We have isolated a partial cDNA clone for CBB-MI that encodes the C-terminal ~35 kDa of the heavy chain. The sequence of this clone is identical to that of an authentic, near-full-length CBB-MI cDNA clone reported recently, except for an 87-bp/29-residue insertion occurring ~32 kDa from the C-terminus. This insert, which is probably generated by an alternate splicing event, is expressed in brush border as part of a message of the size predicted for the CBB-MI heavy chain, although the steady state level of this transcript is ~8-fold lower than for transcripts lacking the insert. ^{125}I-CAM overlays of this cDNA clone (expressed as a trpE fusion protein in *E. coli*) indicate that it binds one more calmodulin than does a second cDNA clone that lacks the 29-residue insert. A synthetic peptide corresponding to the insert sequence binds tightly to CAM-Sepharose, demonstrates a shift and enhancement of fluorescence in the presence of CAM, and binds CAM in solution with a K_D of 190 nM (in 100 mM KCl). We conclude that a second, low-abundance isoform of CBB-MI contains an additional (and possibly fourth) CAM binding site as a result of a 29-residue peptide that is inserted into the tail domain by an apparent alternate splicing event.

Chicken brush border myosin I; Calmodulin binding; Alternate splicing

1. INTRODUCTION

Brush border myosin I (BB-MI), known previously as the brush border 110 kDa protein, is located in the microvillus of the vertebrate intestinal brush border cell where it forms, as a complex with calmodulin, a lateral link between the central actin filament bundle and the plasma membrane (for reviews see [1,2]). This complex, which has been studied most extensively using chicken intestinal tissue as a source, shares many properties with myosins, including an actin-activated Mg^{2+}-ATPase activity [3,4] and mechanochemical activity [5,6]. The sequence of an authentic, near-full-length cDNA clone for the heavy chain of chicken BB-MI reveals a ~119 kDa polypeptide that is composed of an ~82 kDa myosin globular head ('S1') domain fused to a ~37 kDa tail domain that bears no resemblance to the rod-like tail of conventional myosins [7] (see Fig. 1). Peptide mapping studies indicate that the CAM binding sites reside somewhere within the unconventional, ~37 kDa tail domain [8,9].

As isolated, CBB-MI is a complex between one heavy chain and three calmodulin 'light chains' [6]. Recent data reveal that these CAM light chains, together with Ca^{2+}, regulate the ATPase and mechanochemical activities of the protein [5,6]. We present here evidence that a second isoform of CBB-MI, which is probably of relatively low abundance, contains an additional (and possibly fourth) CAM binding site as a result of a 29-residue peptide that is inserted by an apparent alternate splicing event.

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2. MATERIALS AND METHODS

Standard methods were used for library screening and DNA subcloning [10]. The chicken intestinal λgt11 cDNA library and the bovine BB-MI probe were gifts from D. McDonald and S. Nakanishi, respectively. DNA was sequenced using the dideoxy method [11]. Northern and Southern blots were probed with end-labeled oligonucleotides as described [10]. CBB-MI cDNA clones and cDNA clone fragments were expressed in *E. coli* (HB 101) as C-terminal fusions to 37 kDa of the bacterial protein trpE using pATH vectors, as described by Hoffman et al. [12]. Highly enriched fractions of fusion proteins were prepared by differential centrifugation and washing of insoluble pellets in Triton X-100 containing buffers [12]. ^{125}I-calmodulin overlays were performed in the presence of 0.1 mM $CaCl_2$ as described by Slaughter and Means [13] on SDS-polyacrylamide minigels (4–20% gradient). Autoradiograms and parallel gels stained with Coomassie blue were quantified by densitometry. A peptide corresponding to the 29-residue insert was made on a Vega model 250 solid-phase peptide synthesizer and purified by reverse phase HPLC. Purity was assessed by amino acid analysis. For chromatography on CAM:Sepharose (Pharmacia), the resin was washed extensively in 10 mM Tris (pH 7.5), loaded with a half molar equivalent of peptide, washed with 10 bed volumes of buffer, and subjected to a 0–2 M KCl gradient. Some gradients were run in the presence of 2 M urea. All buffers contained either 0.1 mM $CaCl_2$ or

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1 mM EGTA. Elution of the peptide was monitored at 278 nm. Fluorescence measurements of calmodulin:peptide interactions were made using a Perkin-Elmer LS-5 fluorimeter (excitation wavelength, 290 nm) in a buffer containing 10 mM Tris (pH 7.5), 100 mM KCl and 1 μM peptide, and either 0.1 mM $CaCl_2$ or 1 mM EGTA. Calmodulin fluorescence and background fluorescence were corrected for by repeating experiments in the absence of peptide. The binding data in Fig. 3B were directly fitted to a nonlinear least squares fitting routine (DEC-10 MLAB program, DCRT, NIH) that is derived from a single site equilibrium and assumes a linear relationship between fractional saturation and fluorescence enhancement.

3. RESULTS AND DISCUSSION

A ~2 kb *Eco*RI fragment spanning the 3′ half of a cDNA clone encoding the heavy chain of bovine BB-MI [14] was used as a heterologous probe to isolate chicken BB-MI heavy chain cDNA clones from a chicken intestinal λgt11 cDNA library. Two groups of putative chicken BB-MI clones were isolated (groups A and B). Both groups contained three members each, all of which were fully sequenced on both strands. The longest clone in each group, designated clone A and clone B, encompassed all other clones within their respective groups. The nucleotide sequence of clone A was found (i) to be identical to that of authentic chicken BB-MI, determined recently from a near-full-length cDNA clone [7], and (ii) to encode the C-terminal ~35 kDa of the heavy chain (Fig. 1). The sequence of clone B was identical to clone A (and to authentic CBB-MI) except for an 87-bp/29-residue in-frame insertion occurring ~32 kDa from the C-terminus (Fig. 1)[1]. This insertion, whose derived protein sequence is SRRLLRELKVQRRRHLAASTISAY-WKGYQ, falls between amino acid residues 716 and 717 in the published sequence of CBB-MI [7]. The fact that, with the exception of the insertion, the nucleotide sequences of clones A, B and CBB-MI [7] are identical throughout, including the entire 3′ untranslated sequences, lends strong support to the idea that the insertion is a result of alternate splicing. Specifically, the two transcripts represented by clones A and B probably arose from the same gene by alternate splicing of a single primary transcript in such a way that the exon encoding the 29-residue insert is included in one transcript and excluded from the other. Confirmation of this idea must, however, await analyses of chicken BB-MI genomic clones.

To determine if the insert sequence is expressed in chicken BB as part of a message of the size predicted for the 119 kDa CBB-MI heavy chain, and to estimate the relative expression of RNAs in BB that correspond to cDNA clones A and B, a Northern blot of chicken BB RNA was probed with an oligonucleotide that distinguishes clone B from clone A. This clone B-specific oligonucleotide probe (40 nt) encodes a portion of the 29-residue inserted sequence[1] and, as expected, does not cross-hybridize with the DNA insert of cDNA clone A (Fig. 2C, 'insert oligo'). A 40 nt oligonucleotide probe corresponding to nucleotides 2175–2215 of the CBB-MI sequence [7] was also synthesized. This probe is common to both clones A and B, has a similar G+C content to the clone B-specific probe, and hybridizes equally well with the DNA inserts of both cDNA clones (Fig. 2C, 'universal oligo'). These two oligonucleotides were end-labelled to equivalent specific activities and hybridized with chicken BB mRNA (Fig. 2D). Both probes hybridized to a ~3.5 kb transcript, although the signal intensity for the clone B-specific probe is about one eighth of that for the universal probe. These results show that the 29-residue insert is expressed in brush border as part of a low-abundance message of the size expected for the CBB-MI heavy chain.

The tail domain of CBB-MI is thought to contain the CAM binding sites [8,9]. To determine whether the 29-residue insert might contain a CAM binding site, clones A and B were expressed in *E. coli* as fusions to a 37 kDa fragment of the *E. coli* protein trpE. Also expressed were three different 5′-end deletions (DEL1-DEL3), in which successively larger regions from the N-terminus of clone B were deleted. Fig. 1 shows the nature of these N-terminal deletions in schematic form. All 5 fusion proteins were produced in *E. coli* as insoluble aggregates, which were purified by differential centrifugation and detergent washing. SDS-PAGE analysis of the resultant pellets revealed fractions that were highly enriched in the appropriately-sized fusion protein (Fig. 2A).

To examine CAM binding by these fusion proteins, an ^{125}I-CAM overlay assay was used [13]. SDS-polyacrylamide gels of the 5 fusion proteins were overlaid with ^{125}I-CAM, washed, processed for autoradiography, and the relative amounts of CAM bound were estimated by densitometric scanning of the resultant autoradiograms. The values obtained were normalized to the clone-A fusion peptide value by correcting for differences in the amounts of protein loaded. This was done by densitometric scanning of parallel, Coomassie blue-stained gels, coupled with adjustment for the slight differences in the lengths of the proteins. Fig. 2B shows a typical result (this autoradiogram was from an overlay performed on a gel that was loaded exactly like the gel shown in Fig. 2A), while Table I gives the normalized data averaged from 3 separate experiments. The most striking finding is that the clone-B fusion protein bound 2.2 times as much CAM as the clone-A fusion protein. This difference could be due to an increase in the affinity of a neighboring CAM interaction site as a result of the in-

[1] The nucleotide sequence of this insert, which was found in three independent group B cDNA clones, is 5′ TCCCGCCGGCTCCT-TCGGGAGCTGAAGGTTCAGCGCCGCCGTCATTTGGCCG-CCAGCACCATTTCTGCATACTGGAAAGGGTATCAG 3′. The insert-specific oligo was the last 40 nucleotides of this sequence

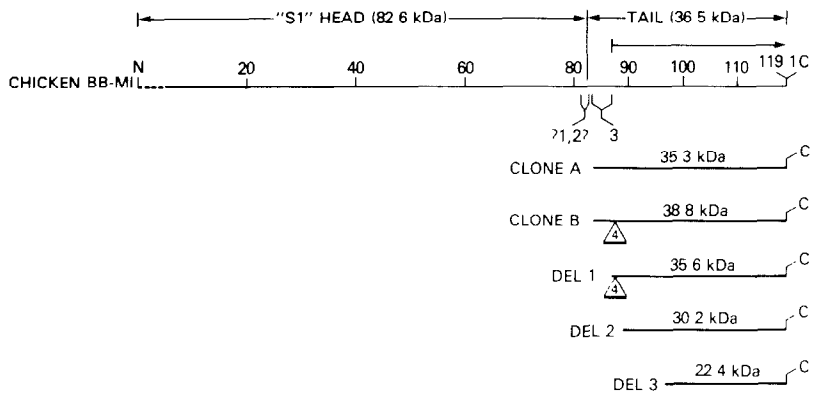

Fig 1. Schematic relating clones A and B and deletions 1–3 to the 119 kDa heavy chain of CBB-MI. Data concerning the whole heavy chain are from [7]; an extra 5 kDa was added at the N-terminus of their near-full-length sequence, based on alignments with the full-length sequence of bovine BB-MI [14]

sertion of the 29-residue sequence, or to the presence within the 29-residue insert of an additional CAM interaction site. Analysis of a synthetic peptide corresponding to the 29-residue insert (see below) supports the latter explanation. The results are consistent, therefore, with clone B containing two CAM binding sites, one of which is in common with clone A (referred to as site 3 in Fig. 1) and one of which is unique to clone B and present in the insert sequence (site 4 in Fig. 1). Regarding the location of the common site, we found that the DEL1 fusion protein, which lacks the sequence N-terminal to the insert (which is common to clones A and B), bound only one half as much CAM as the intact clone-B fusion protein (see Table I). This result is consistent with the common site residing in the DEL1 difference peptide, i.e. between 32 kDa and 35 kDa from the C-terminus (or 36–39 kDa for the isoform containing the insert sequence).

The overlay data also indicated that the DEL2 and DEL3 fusion proteins do not bind CAM (even longer autoradiographic exposures than shown in Fig. 2B showed no binding). These results indicate that the C-terminal most ~30 kDa of the CBB-MI heavy chain does not contain any CAM binding sites. These results also serve as a control by indicating that CAM does not bind to the trpE portion of the fusion proteins. If, as is likely, the near-full-length CBB-MI clone [7] (and hence clone A) encodes the major isoform of CBB-MI, and since this isoform is known to bind 3 mol of CAM/mol heavy chain, then two additional CAM binding sites must be N-terminal of clone A. Indeed, computer searches using the program of Erickson-Viitanen and Delgrado [15] (data not shown and [16]) identified two potential CAM binding sites at the head/tail junction (sites 1 and 2 in Fig. 1), which is only ~2 kDa N-terminal of clone A.

To determine directly whether the positively charged, 29-residue insert contains a CAM binding site, a peptide corresponding to the insert was synthesized. The first evidence that this peptide contains a CAM binding site was that it bound to a CAM:Sepharose column. Binding was not Ca^{2+} dependent, however, as the peptide bound in 1 mM EGTA as well as in 0.1 mM $CaCl_2$. Furthermore, the peptide could not be eluted in the presence or absence of Ca^{2+} using a salt gradient of 0–2 M KCl. Similar experiments using a peptide derived from myosin light chain kinase [17] showed Ca^{2+} sensitive elution from CAM:Sepharose only in the presence of 2 M urea. Therefore, 2 M urea was added to the salt gradients and the experiments repeated. Greater than 90% of the peptide eluted as a sharp peak at ~150 mM KCl, 2 M urea in the absence of Ca^{2+}. Less than 5% of the peptide eluted with 2 M KCl, 2 M urea in the presence of Ca^{2+}. Therefore, while the interaction between the peptide and CAM:Sepharose is not strictly Ca^{2+} dependent, some Ca^{2+} sensitivity is seen.

To characterize further the interaction between CAM and the peptide, we used fluorescence spectroscopy, which was possible because the insert peptide contains a tryptophan (and CAM does not) [18]. Fig. 3A (tracing A) shows the emission spectra of the peptide alone (peak 355 nm). Superimposable tracings were seen in the presence and absence of Ca^{2+} and when an equimolar amount of CAM was added to the peptide in the absence of Ca^{2+} (100 mM KCl). In the presence of Ca^{2+}, however, an interaction between the peptide and CAM was apparent in an enhancement of fluorescence and a shift in the emission peak to 327 nm (Fig. 3A, tracing B). Very similar emission shifts have been described [15,18] and reflect the fact that the tryptophan residue shifts to a hydrophobic environment upon peptide:CAM interaction [18]. To determine the approximate stoichiometry of the CAM:peptide interaction and the binding affinity, the effect of adding increasing amounts of CAM on peptide fluorescence at 327 nm was determined in the presence of Ca^{2+} (Fig. 3B). Fluorescence enhancement was observed to

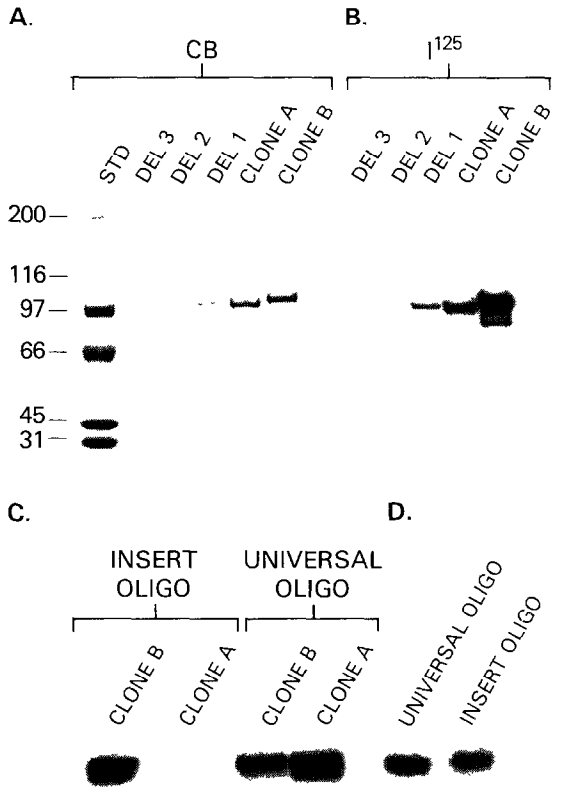

Fig. 2. (A) Coomassie blue (CB) stain of the trpE fusion protein fractions. (B) Autoradiogram of an ^{125}I-CAM overlay performed on the gel in (A). (C) Southern blots demonstrating the specificity of the universal and insert-specific probes (shown are the hybridizations with 1 μg of the *Eco*RI inserts of cDNA clones A and B). (D) Northern blots of CBB RNA (15 μg of total RNA per lane) probed with the universal (16 h exposure) and insert-specific (40 h exposure) oligonucleotides (shown is the ~3.5 kb transcript detected by these probes).

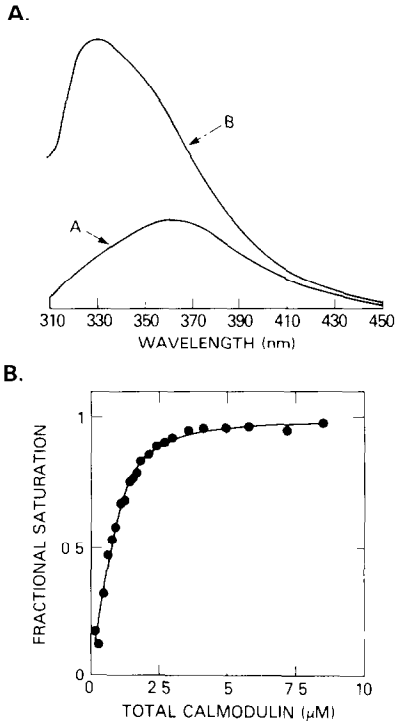

Fig. 3. (A) Fluorescence shift and enhancement accompanying peptide-CAM interaction. (B) Dependence of peptide fluorescence on CAM concentration.

be hyperbolically dependent on CAM concentration. The data were fitted directly to a hyperbola as described in section 2 and indicate a single CAM binding site with a dissociation constant of 190 nM (in 100 mM KCl). This value falls within the range of previously reported affinities for CAM binding proteins, but is ~10-fold lower than the average for a number of previously characterized peptides that bind CAM [18]. We note that at low ionic strength (10 mM KCl), addition of CAM to the 29-residue peptide in the absence of Ca^{2+} caused an enhancement of fluorescence without a shift in the emission peak (data not shown). Similar results, i.e. Ca^{2+}-independent, CAM-induced fluorescence enhancement that is abolished at higher ionic strength, have been reported for a synthetic peptide from neuromodulin [19].

In conclusion, the data presented here indicate that the 29-residue insert contains a CAM binding site, which may have important implications for the regulation of CBB-MI ATPase activity and mechanochemical properties. Furthermore, the data provide concrete evidence for multiple protein isoforms of CBB-MI and an example of a CAM binding site occurring within an alternately spliced exon. Antibodies directed against the 29-residue sequence should facilitate the purification of this second CBB-MI isoform.

Table I

^{125}I-CAM binding results

Clone A	1.0
Clone B	2.2 ± 0.4
DEL1	1.0 ± 0.3
DEL2	0
DEL3	0

These values of relative CAM binding were normalized to the value for the clone A-fusion protein (n = 3).

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