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Genetic evidence that *Acanthamoeba* myosin I is a true myosin

(low molecular weight nonmuscle myosin/gene isolation/sequence homology/conventional myosins)

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ABSTRACT **_Acanthamoeba castellanii_ contains two enzymes, myosins IA and IB, that exhibit the catalytic properties of a myosin but possess very unusual physical properties, the most striking of which are their single, low molecular weight heavy chain, their globular shape, and their inability to form bipolar filaments. We have now isolated a putative myosin IB heavy chain gene from _Acanthamoeba_, using as a heterologous probe a portion of a sarcomeric myosin heavy chain gene from _Caenorhabditis elegans_. The amoeba genomic clone hybridizes to a 4250-nucleotide RNA species and hybrid-selects an mRNA encoding a 125-kDa polypeptide. This polypeptide comigrates exactly with the heavy chain of purified amoeba myosin IB and is specifically immunoprecipitated with antiserum to myosin IB. We sequenced two restriction enzyme fragments of this gene, and the deduced amino acid sequences show strong homology with the regions of muscle myosins that contain the reactive thiols and the ATP binding site. Our identification of a myosin IB heavy chain gene demonstrates that myosin IB, despite the unusually low molecular weight of its heavy chain, is a true gene product. The sequence results show that, despite its atypical physical properties, myosin IB is clearly related to conventional myosins.**

Myosins from both muscle and nonmuscle sources are hexameric proteins, containing a pair of ≈200-kDa heavy chains and two pairs of ≈20-kDa light chains (for review, see ref. 1). These subunits are arranged in a highly asymmetric molecule possessing two globular head regions and a rod-like tail made of a pair of intercoiled α-helices. The rod portion mediates the self-assembly of myosin monomers into bipolar myosin filaments, the functional form of the enzyme. The globular heads contain the actin binding region, the actin-activated ATPase catalytic site, and the light chain binding sites. The globular heads project from the surface of the myosin filament and undergo cyclic interactions (cross-bridges) with neighboring actin filaments to generate contractile force (1).

Three enzymes with myosin-like catalytic activities and actin-binding properties have been purified from the soil amoeba *Acanthamoeba castellanii*. One of these, myosin II, is a structurally typical myosin, being composed of a pair of 185-kDa heavy chains and two pairs of light chains (17 kDa and 17.5 kDa) (2). It has the α-helical coiled-coil tail that mediates self-assembly into bipolar filaments and globular heads with actin-activated ATPase activity (3, 4). On the other hand, *Acanthamoeba* myosins IA and IB are structurally quite different. Each consists of only a single heavy chain, which is small compared to conventional myosins (130 kDa and 125 kDa, respectively), and a single light chain (17 kDa and 27 kDa, respectively) (5). Based on hydrodynamic properties, electron microscopic images, and circular dichroism spectra, myosins IA and IB have been shown to be roughly globular, monomeric molecules that lack the extended rod-like tail and are incapable of self-assembly into filaments or even into small oligomers (6).

The only well-developed model for actomyosin-dependent contractile and motile activities is the sliding-filament model, which was deduced from the properties of skeletal muscle actomyosin (7, 8). This model depends specifically on the ability of myosin to form bipolar filaments and, in detail, on the precise structural reorientations between the head and the rod portions of the myosin molecule during the crossbridge cycle that is coupled to the hydrolysis of ATP. However, actomyosins IA and IB can support analogues of contractile and motile activity *in vitro* (6, 9). Specifically, both myosins IA and IB cause superprecipitation of F-actin dependent on ATP hydrolysis (9). In addition, both myosins cause beads to which they are attached to move in a unidirectional, ATP-hydrolysis-dependent manner on oriented cables of *Nitella* actin *in vitro* (6). Therefore it becomes very important, not only for their own intrinsic interest but also for the understanding of actomyosin function in general, to establish whether these nonfilamentous, monomolecular, actin-activated ATPases are truly myosins and, if so, that they are not degradation products of larger, structurally more typical myosins.

We recently reported the isolation of a genomic clone containing an *Acanthamoeba* myosin II heavy chain gene (10). This cytoplasmic myosin gene was identified by using as a heterologous probe a portion of the sarcomeric *unc-54* myosin heavy chain gene from the nematode *Caenorhabditis elegans* (11). In analyzing additional amoeba genomic clones that were positive with this heterologous probe, we have identified a gene encoding the 125-kDa heavy chain of a myosin IB. The results in this paper demonstrate that, despite its small size and other atypical physical properties, the myosin IB heavy chain is a true gene product and a true myosin.

MATERIALS AND METHODS

All methods, including blot hybridizations, construction of the *Acanthamoeba* genomic library in phage λ2001, screening of the library with the heterologous nematode probes, hybrid-selection analysis, and DNA sequencing by the dideoxynucleotide method in phage M13 were performed exactly as described previously (10). The immunoprecipitation reactions, the competition immunoprecipitation reactions, and sodium dodecyl sulfate/polyacrylamide gel electrophoresis and fluorography were as described (12). The recombinant λ1059 phage containing the nematode *unc-54* myosin heavy chain gene (13) and the λ2001 cloning vector were generous gifts of J. Karn and L. Barnett (Medical Research Council Centre, Cambridge, U.K.). Myosins IA and IB were gifts of J. P. Albanesi, H. Fujisaki, and T. Lynch (Laboratory of Cell Biology, National Heart, Lung, and Blood Institute). Myosin IB polyclonal antiserum was prepared as described (12). The synthetic oligonucleotides used as primers for DNA sequenc-

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Abbreviations: bp, base pair(s); kb, kilobase(s).

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RESULTS

Isolation of Acanthamoeba Genomic Clone λ3·9. To search for *Acanthamoeba* myosin heavy chain genes, we used as a heterologous probe a 2830-base-pair (bp) *Bam*HI fragment from the nematode *unc-54* myosin heavy chain gene (11). This fragment contains 2181 bp of coding sequence interrupted by four intervening sequences of 479, 79, 53, and 38 bp (11). The fragment encodes amino acid residues 35–761, or ≈90% of the nematode myosin globular head, including the actin- and ATP-binding sites and the reactive cysteine residues. We previously found that this nematode probe hybridized to eight amoeba DNA restriction fragments [totaling nearly 60 kilobases (kb) of DNA] in a Southern blot of *Bam*HI-digested amoeba genomic DNA (10). The nematode probe also recognized two high molecular weight RNAs (5300 and 4250 nucleotides) in a blot of amoeba poly(A)$^+$ RNA (Fig. 1, lane 1). Based on these results, we decided to search directly for *Acanthamoeba* myosin heavy chain genes by screening a genomic library of *Acanthamoeba* DNA with this nematode probe. The construction and screening of this phage genomic library have been described (10). In brief, we screened about 40 genome equivalents (80,000 plaques) with the nematode probe and obtained ≈4 positive phage per genome equivalent. Forty positive phage clones were picked at random and plaque-purified. These were rescreened with a subfragment of the nematode probe, a 490-bp *Ava* I–*Bam*HI piece that encodes amino acids 598–761. This region contains the two reactive cysteines at positions 705 and 715. This "reactive thiol" region is highly conserved in myosins (11, 14). Restriction mapping of the seven phage clones that were positive for this "reactive thiol" probe yielded two groups of overlapping phage (three in one group, two in the other) and two individual, nonoverlapping phage. The group containing three members has been shown to contain an amoeba myosin II heavy chain gene (10). Here we show that one of the individual, nonoverlapping phage clones, genomic clone λ3·9, contains a myosin IB heavy chain gene. Fig. 2*A* shows the restriction enzyme map of phage clone λ3·9. We found that the rightward 5.3-kb *Bam*HI–*Xba* I fragment in the λ3·9 insert hybridized to a 4250-nucleotide amoeba RNA species (Fig. 1, lane 2). This RNA comigrated exactly with the 4250-nucleotide RNA species recognized by the 2.8-kb nematode probe (Fig. 1; compare lanes 1 and 2). Further, the insert DNA in λ3·9 to the left of the *Hin*dIII site did not hybridize to the 4250-nucleotide RNA species (data not shown), suggesting that the coding information for the gene was localized to the right-most 4 kb of λ3·9 insert DNA. Therefore, the 5.3-kb *Bam*HI–*Xba* I fragment was subcloned in the plasmid pUC12 and a more detailed restriction enzyme map was obtained (Fig. 2*B*).

Hybrid-Selection Analysis. Amoeba genomic clone λ3·9 was identified as containing a myosin IB heavy chain gene by hybrid-selection analysis (Fig. 1, lanes 3–10). Comparison of the [^{35}S]methionine-labeled proteins translated *in vitro* from

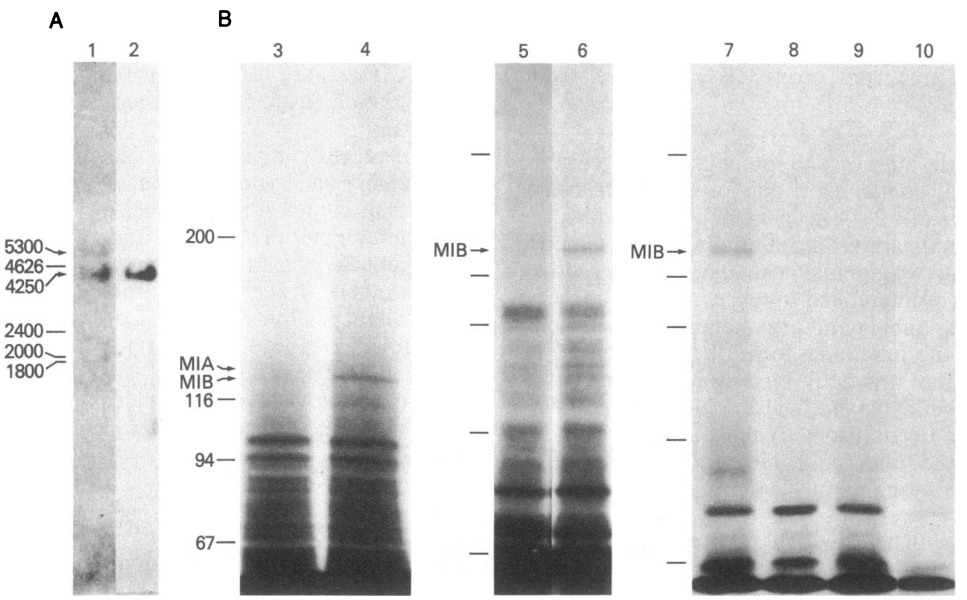

FIG. 1. RNA blot and hybrid-selection analyses of amoeba genomic clone λ3·9. (*A*) RNA blot hybridization. Amoeba poly(A)$^+$ RNA (6 μg) was electrophoresed, blotted, and probed with the 2.8-kb nematode probe (lane 1) or with the 5.3-kb *Bam*HI–*Xba* I fragment from amoeba genomic clone λ3·9 (lane 2) (see Fig. 2 for the location of this fragment in clone λ3·9). The same blot strip was used for both probes. The four marker RNA species were electrophoresed in an adjacent lane and are chicken 28S RNA (4626 nucleotides), the 2400- and 2000-nucleotide pieces of amoeba 26S RNA, and a fused doublet of chicken and amoeba 18S RNA (1800 nucleotides). The previously isolated amoeba myosin II gene hybridized to a 5300-nucleotide mRNA that comigrated exactly with the 5300-nucleotide RNA species recognized by the nematode probe (10). (*B*) Hybrid-selection analysis. Lanes 3 and 4: fluorogram of a $NaDodSO_4$/5% polyacrylamide gel of the [^{35}S]methionine-labeled proteins synthesized *in vitro* from mRNA selected by λ2001 vector DNA (lane 3) or by phage clone λ3·9 DNA (lane 4). Lanes 5 and 6: fluorogram of a 6.8% gel of the labeled proteins synthesized from mRNA selected by plasmid pUC12 DNA (lane 5) or by the 5.3-kb *Bam*HI–*Xba* I pUC12 subclone DNA (lane 6). Lanes 7–9: fluorogram of a 7% gel of the labeled proteins immunoprecipitated from the 5.3-kb *Bam*HI–*Xba* I pUC12 DNA-selected material, using a polyclonal antiserum to myosin IB (lane 7), using the antiserum to myosin IB in an immunoprecipitation reaction to which 20 μg of purified, nonradioactive myosin IB had been added previously (lane 8), and using nonimmune serum (lane 9). Lane 10: fluorogram of a 7% gel of the labeled proteins immunoprecipitated from the control pUC12 DNA-selected material, using the polyclonal antiserum to myosin IB. The positions where the Coomassie blue-stained, authentic 125-kDa myosin IB heavy chain (MIB) [and the 130-kDa myosin IA heavy chain (MIA), in lanes 3 and 4] migrated in an adjacent lane are indicated. When the material in lane 6 was run in a lower percentage acrylamide gel (5%) so as to clearly resolve the myosin IA and IB heavy chain standards, we saw, as in lane 4, no evidence for selection of the myosin IA heavy chain mRNA. Lanes 3 and 4 were 6-hr exposures; lanes 5 and 6, 4-hr exposures; and lanes 7–10, 24-hr exposures. The standards were skeletal muscle myosin heavy chain (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), and actin (42 kDa; run off the gel in lanes 3 and 4).

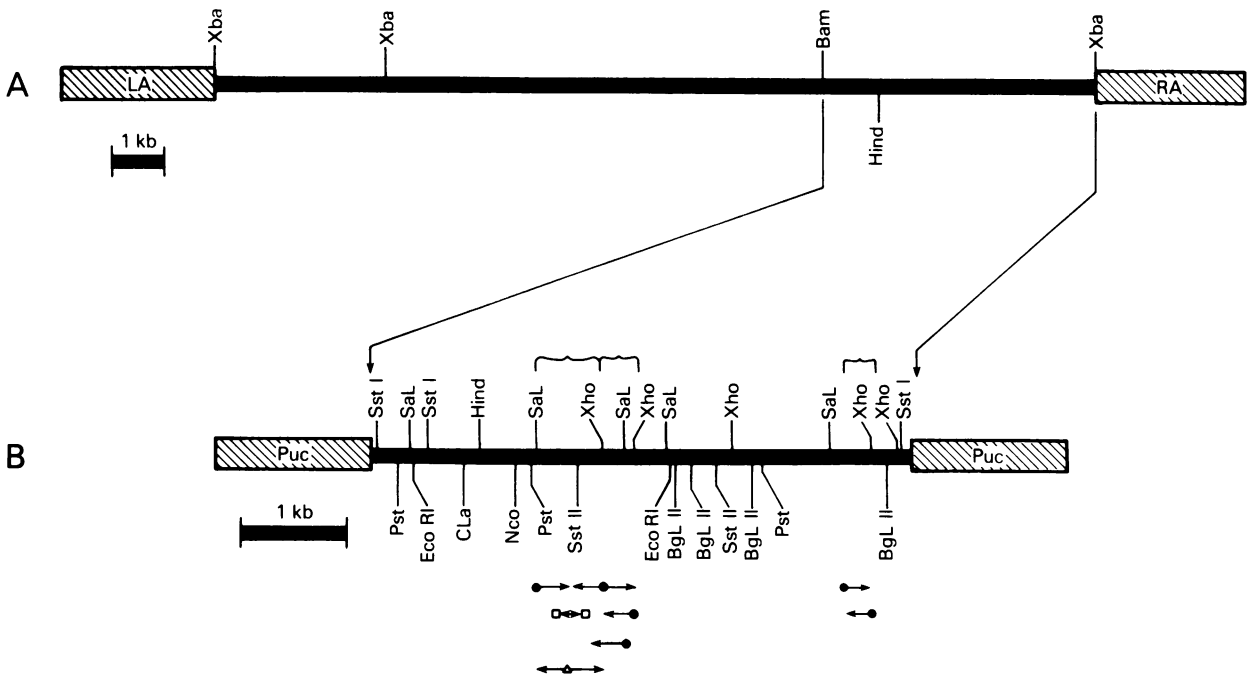

FIG. 2. Restriction enzyme maps of *Acanthamoeba* genomic clone λ3·9. (*A*) Amoeba genomic clone λ3·9 was mapped with *Bam*HI, *Hin*dIII, and *Xba* I. The left and right arms of the phage vector are designated LA and RA, respectively, and both contain an *Xba* I site adjacent to the insert. (*B*) The 5.3-kb *Bam*HI–*Xba* I fragment of λ3·9 was subcloned in pUC12 and mapped with *Bgl* II, *Cla* I, *Eco*RI, *Nco* I, *Pst* I, *Sal* I, *Sst* I, and *Xho* I. The 684-bp *Sal* I–*Xho* I and 258-bp *Xho* I fragments that hybridized with the ATP-binding-site fragment from the amoeba myosin II gene, as well as the 300-bp *Ava* I–*Sau*96I fragment that hybridized with the reactive-thiol region-encoding fragment from the nematode *unc-54* myosin gene, are indicated by braces. Arrows indicate the direction and extent of the areas that were sequenced. Solid circles indicate sequences originating from mapped restriction sites; open boxes, sequences originating from two *Sau*3A sites; open triangle, sequences obtained using synthetic 17-bp oligonucleotides to prime for opposite-strand sequencing. The 300-bp *Ava* I–*Sau*96I fragment was blunt-ended and inserted into the M13 *Sma* I site. The restriction maps above are written left to right in the 5′ to 3′ direction relative to the orientation of the myosin IB heavy chain gene.

mRNA hybrid-selected by the control DNA (phage vector DNA only, lane 3) and the λ3·9 DNA (lane 4) revealed a 125-kDa protein that was unique to the λ3·9 DNA selection. This radioactive protein comigrated exactly with the heavy chain of authentic, purified myosin IB. The same results were obtained when the 5.3-kb *Bam*HI–*Xba* I pUC12 subclone DNA was used for hybrid-selection analysis (lane 5, pUC12 vector DNA only; lane 6, 5.3-kb *Bam*HI–*Xba* I pUC12 subclone DNA). Further, this 125-kDa radioactive protein was specifically immunoprecipitated by a polyclonal antiserum to myosin IB (lane 7). Addition of excess purified nonradioactive myosin IB to the immunoprecipitation reaction prior to the addition of myosin IB antiserum blocked the immunoprecipitation of the 125-kDa protein (lane 8). The protein was not precipitated by nonimmune serum (lane 9), nor was a 125-kDa protein precipitated from the control DNA selection using the myosin IB antiserum (lane 10).

Amino Acid Sequence Homology with Conventional Myosins. We probed restriction enzyme digests of the 5.3-kb *Bam*HI–*Xba* I pUC12 subclone with a 688-bp *Bst*EII/*Pst* I fragment from the previously isolated *Acanthamoeba* myosin II heavy chain gene (10). This 688-bp fragment contains the coding information for residues 101–246 of the 185-kDa myosin II heavy chain (10). This region of the polypeptide is thought to be involved in the binding of ATP, based on chemical crosslinking with a nucleotide analog (15) and on homology with the putative nucleotide-binding-site region of other sequenced myosins (10, 11, 16). This myosin II gene fragment hybridized to two contiguous restriction fragments in the 5.3-kb *Bam*HI–*Xba* I pUC12 subclone digests, a 684-bp *Sal* I–*Xho* I fragment and a 258-bp *Xho* I fragment (data not shown). Fig. 2*B* indicates the position of these two contiguous fragments within the myosin IB gene. Fig. 3*A* shows the nucleotide sequence of these two fragments and the myosin IB amino acid sequence encoded by this region, which was deduced by homology with the amoeba myosin II sequence (Fig. 4*A*). The deduced myosin IB amino acid sequence shows a 48% exact match with residues 91–265 of the amoeba myosin II sequence. Taking into consideration conservative amino acid changes (17), the homology with myosin II is 69%. The deduced myosin IB amino acid sequence also shows a 46% exact match with residues 86–271 of the nematode *unc-54* myosin and a 44% exact match with residues 87–272 of rabbit skeletal muscle myosin (Fig. 4*B*). Taking into consideration conservative residue changes, the homology is 68% and 66% with the nematode and rabbit myosins, respectively. As is the case for myosin II, this region of the nematode and rabbit myosin amino acid sequence falls in the amino-terminal 5–15% of the polypeptide and is thought to be involved in the binding of ATP, as determined by conformational analysis of the sequence (11, 16), sequence homology with other ATP-binding proteins (11), and chemical crosslinking with nucleotide analogs (18–20). The homologous myosin IB sequence appears to be similarly located near the amino terminus, based on the RNA blot results described above.

We also probed restriction enzyme digests of the 5.3-kb *Bam*HI–*Xba* I pUC12 subclone with the 490-bp *Ava* I–*Bam*HI nematode myosin gene fragment, which encodes the conserved reactive thiol region and which identified the myosin IB clone in the screening of the genomic clones. Fig. 2*B* indicates the position of a 300-bp *Ava* I–*Sau*96I fragment, within the myosin IB gene, that hybridized strongly with the thiol probe (data not shown). Fig. 3*B* shows the nucleotide sequence of this fragment and the myosin IB amino acid sequence encoded by this region, which was deduced by homology with the nematode *unc-54* myosin sequence (Fig. 4*C*). The deduced myosin IB amino sequence shows a 40%

FIG. 3. Nucleotide sequence and deduced amino acid sequence of the putative ATP-binding-site region and thiol-containing region of amoeba myosin IB. (*A*) The contiguous 684-bp *Sal* I–*Xho* I and 258-bp *Xho* I fragments were sequenced as described in Fig. 2*B*. The reading frame was deduced by homology with the amino acid sequence of the amoeba myosin II heavy chain (see Fig. 4*A*). (*B*) The 300-bp *Ava* I/*Sau*96I fragment was sequenced as described in Fig. 2*B*. The reading frame was deduced by homology with the amino acid sequence of the nematode *unc-54* myosin (see Fig. 4*C*). In both *A* and *B*, the pertinent restriction sites and the positions of the apparent introns are shown. The 5′ donor (GT) 3′ acceptor (AG) splice sites are boxed. The standard one-letter abbreviations are used for amino acid residues.

exact match with residues 636–741 of the nematode myosin. Taking into consideration conservative amino acid changes, the homology is 62% with the nematode myosin sequence. Interestingly, the myosin IB sequence does not contain cysteine residues in either of the positions of the nematode myosin active thiols.

The myosin IB sequence (Fig. 3*A*) is interrupted by four small apparent introns of 123, 88, 91, and 130 bp. These introns were identified because (*i*) they interrupt contiguous stretches of myosin IB coding sequence that are highly homologous to the sequences of other myosins, (*ii*) they have 5′ donor and 3′ acceptor splice sites that conform to the GT...AG rule (21), and (*iii*) they are unusually pyrimidine-rich.

DISCUSSION

The data essentially rule out the possibility that genomic clone λ3·9 contains a myosin II heavy chain gene, since (*i*) the clone hybridizes to an RNA species (4250 nucleotides) that is too small to encode the 185-kDa myosin II heavy chain [which would require a minimum of ≈5000 nucleotides (10)], (*ii*) the clone hybrid selects an mRNA that encodes a much smaller polypeptide (125 kDa) than the 185-kDa myosin II heavy chain, and (*iii*) the 125-kDa polypeptide is specifically immunoprecipitated with a myosin IB antiserum that does not crossreact with myosin II (12). Rather, the hybrid selection and sequence data fit closely the expected properties of the low molecular weight heavy chain of myosin IB—i.e., a 125-kDa polypeptide containing sequences characteristic of the ATP-binding-site/reactive-thiol region of other myosin heavy chains. However, we cannot be certain that clone λ3·9 encodes the heavy chain of the specific myosin IB enzyme previously isolated and characterized, rather than a closely related isoenzyme.

Acanthamoeba myosin IB possesses very unusual physical properties for a myosin, raising the possibility that it might have been completely unrelated at the primary-structure level to conventional myosins. However, the myosin IB amino acid sequence deduced thus far, which spans two separate regions (representing a total of ≈20% of the heavy chain), shows strong homology with portions of the globular head regions of three structurally typical myosins: nematode myosin, rabbit skeletal muscle myosin, and *Acanthamoeba* myosin II. The alignment of the sequences is almost exact, except for several gaps in the myosin IB sequence relative to the other myosins. These "missing" amino acids fall at or very near the sites where, in other myosins (11, 16), the globular head region has been shown to be cleaved by limited proteolysis with trypsin into three characteristic fragments (≈23 kDa, ≈50 kDa, and ≈20 kDa, aligned in this order within the heavy chain polypeptide). Specifically, the 5-residue gap in the myosin IB ATP-binding-site sequence relative to the amoeba myosin II sequence (Fig. 4*A*) and the analogous 16-residue gap in the same myosin IB sequence relative to the nematode and rabbit sequences (Fig. 4*B*) occur at the 23-kDa/50-kDa tryptic cleavage site [rabbit lysines 205–207 (16)]. Similarly, the adjacent 1- and 5-residue gaps in the myosin IB thiol-containing region relative to the nematode sequence (Fig. 4*C*) occur very near the 50-kDa/20-kDa tryptic cleavage site [predicted to be one of several lysine residues between nematode residues 637 and 650 (11)]. These tryptic cleavage sites are thought to occur at lysine-rich surface loops in the native myosin head (11). It is known that insertion and deletion of amino acids during the divergent evolution of two homologous proteins most readily occur in surface-loop regions (22). Further, comparison of the head sequences of two nematode myosin heavy chains, which overall are 82.4% invariant, reveals both high sequence variability as well as small gaps in the immediate area of these putative surface loops (11).

Recently, Strehler *et al.* (23) reported that the positions of several of the introns in the 5′-end region of the nematode *unc-54*, rat α cardiac, and rat embryonic myosin heavy chain genes are conserved. Those authors concluded that the conserved interruptions of the myosin heavy chain genes suggest the existence of a highly split ancestral myosin gene from which different lineages removed and/or added specific sets of introns (23). Similarly, we observed that the 123-bp myosin IB intron (see Fig. 3*A*) interrupts a highly conserved

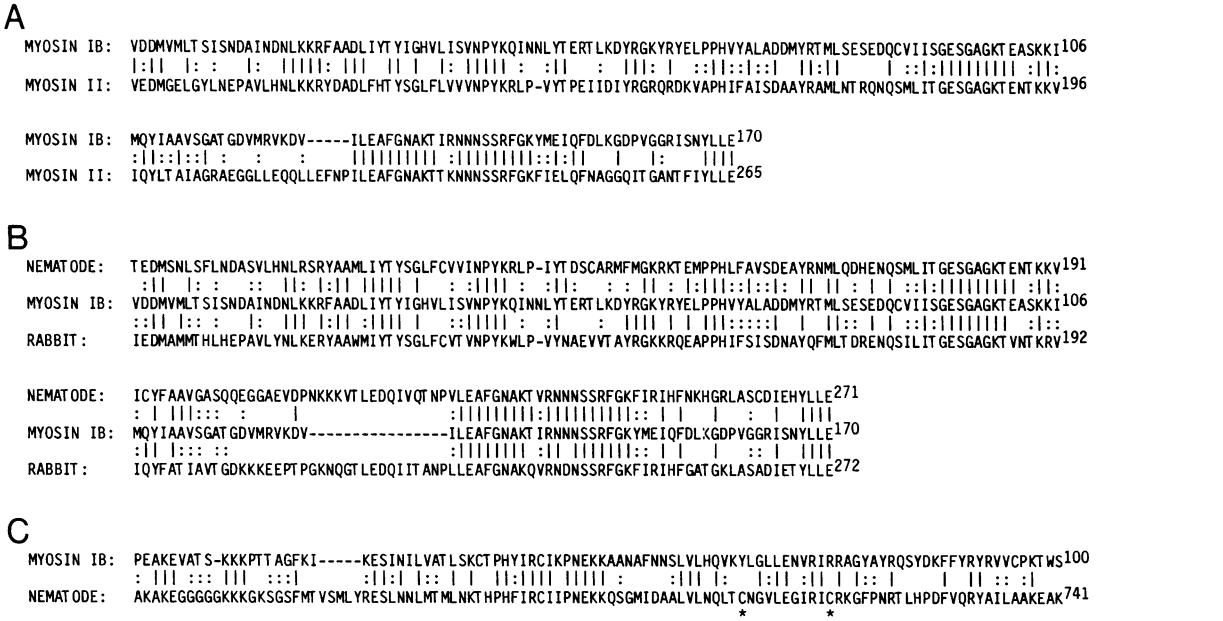

FIG. 4. Amino acid sequence comparisons of the ATP-binding-site regions and the thiol-containing regions in amoeba myosin IB, amoeba myosin II, nematode *unc-54* myosin, and rabbit skeletal muscle myosin. (*A*) Comparison of the deduced myosin IB amino acid sequence from Fig. 3*A* with the putative ATP-binding-site region of amoeba myosin II (ref. 10; the first 5 residues of the myosin II sequence are unpublished results). (*B*) Comparison of the deduced myosin IB amino acid sequence from Fig. 3*A* with the putative ATP-binding-site regions of nematode (11) and rabbit (16) myosin sequences. (*C*) Comparison of the deduced myosin IB amino acid sequence from Fig. 3*B* with the reactive thiol-containing region of nematode myosin (the reactive nematode cysteines are marked with asterisks) (11). The numbering for the amoeba myosin II, nematode myosin, and rabbit myosin refers to residue 1 as the amino terminus of the proteins. The numbering of the myosin IB sequences is arbitrary. Bars indicate an exact match and colons indicate a conservative amino acid change. Several gaps (dashes) in the sequences were introduced to maximize the homology (see *Discussion*). The percent homology presented in the text was calculated based on the total number of possible matches and was therefore corrected for the gaps between the sequences.

amino acid sequence in exactly the same location as does an intron in the 5'-end region of all three myosin genes analyzed by Strehler *et al.* (23). Further, both the 123-bp and 91-bp myosin IB intron positions correspond exactly to the positions of two 5'-end introns in the amoeba myosin II gene (10). These observations also suggest that the myosin IB gene is related to the genes of conventional myosins by a common ancestor.

The results presented here indicate that the purified form of *Acanthamoeba* myosin IB, despite possessing an unusually small heavy chain, is truly representative of the molecule *in vivo*. The results also indicate that myosin IB, despite its unusual physical properties, is closely related to conventional myosins, at least in the portion of the heavy chain that folds to form the globular head. Eventually, comparison of the complete myosin IB amino acid sequence to the known sequence of muscle myosins should provide insight into the structural requirements for myosin function. In addition, it is important to know whether myosin I-like enzymes are widely distributed in nature. Recently, Cote *et al.* (24) purified a myosin I-like enzyme from the slime mold *Dictyostelium discoideum*. The isolation of an *Acanthamoeba* gene that encodes a myosin IB heavy chain will allow us to search at the DNA level for similar proteins in higher eukaryotes.

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