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PRIMARY PEPTIDE SEQUENCES FROM SQUID MUSCLE AND OPTIC LOBE MYOSIN IIs: A STRATEGY TO IDENTIFY AN ORGANELLE MYOSIN

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Abstract

The squid giant axon provides an excellent model system for the study of actin-based organelle transport likely to be mediated by myosins, but the identification of these motors has proven to be difficult. Here the authors purified and obtained primary peptide sequence of squid muscle myosin as a first step in a strategy designed to identify myosins in the squid nervous system. Limited digestion yielded fourteen peptides derived from the muscle myosin which possess high amino acid sequence identities to myosin II from scallop (60–95%) and chick pectoralis muscle (31–83%). Antibodies generated to this purified muscle myosin were used to isolate a potential myosin from squid optic lobe which yielded 11 peptide fragments. Sequences from six of these fragments identified this protein as a myosin II. The other five sequences matched myosin II (50–60%, identities), and some also matched unconventional myosins (33–50%). A single band that has a molecular weight similar to the myosin purified from optic lobe copurifies with axoplasmic organelles, and, like the optic lobe myosin, this band is also recognized by the antibodies raised against squid muscle myosin II. Hence, this strategy provides an approach to the identification of a myosin associated with motile axoplasmic organelles.

Keywords

myosin; squid; organelle; axoplasm; muscle

INTRODUCTION

Myosin motors mediate movement along actin filaments, as in muscle contraction or in the movement of organelles on actin. Our goal is to design a strategy to obtain molecular tools for the investigation of a particular myosin (p235) associated with axoplasmic vesicles in the squid (Bearer *et al.*, 1993, 1996b). To this end, we first tried to obtain amino acid sequences from this myosin, a >220-kDa protein copurifying with axoplasmic organelles (Bearer *et al.*,

1996a). Although we were successful in obtaining the primary amino acid sequence of an associated small light chain, which allowed us to clone it (Bearer *et al.*, 1996a), we did not obtain sequences from the much larger heavy chain, because of limitations in the amount of protein that could be obtained from axons. More protein is needed to sequence a large myosin than for other smaller proteins because myosins are relatively protease resistant (Bálint *et al.*, 1968), and limited proteolysis of any large protein yields multiple peptides of similar sizes. These peptides must be separated by at least two successive fractionations to achieve sufficient purity for Edman degradative sequencing.

Our strategy (see Fig. 1) was to use squid muscle myosin to develop methods for purification, obtain primary amino acid sequences, and to make broad spectrum anti-myosin antibodies. These tools could then be applied to the study of squid neuronal myosins in optic lobes and the axon. As a first step, we semi-purified myosin II from squid syphon muscle, ending the purification with electrophoretic separation. Since squid (*Loligo pealeii*) muscle myosin heavy chain had not been previously purified, we confirmed that our purified protein was myosin by comparing its structure to that of other well-characterized muscle myosins by glycerol spray electron microscopy (Elliott *et al.*, 1976; Shotten *et al.*, 1979). We then used this myosin as starting material upon which to optimize peptide-sequencing techniques. Finally, we generated antibodies to the full length, two-headed muscle myosin with the hopes of obtaining antibodies that would recognize conserved domains in the heads of neuronal myosins.

We then used the anti-muscle myosin antibodies to identify a myosin and follow it through purification in squid optic lobe homogenates. Application of an optimized peptide-sequencing technique to this myosin produced 11 sequences that definitively identified it. Next we used the anti-myosin antibodies to probe KI-washed axoplasmic organelles, which we had previously shown to be capable of rapid movements on actin filaments (Bearer *et al.*, 1996b). Thus, in this report we describe a strategy to identify a specific myosin associated with organelles. In the process, we have generated a number of peptide sequences, methods, and antibodies that should also be of future use for further studies.

MATERIALS AND METHODS

Myosin purifications

Squid, *Loligo pealeii*, were obtained at the Marine Biological Laboratory in Woods Hole, MA. Mantle muscle myosin was purified according to Konno (1978) from *Loligo pealeii* instead of *Ommastrephes sloani pacificus*, a close relative used in the published purification. The final mantle myosin pellet was stored in myosin storage solution [MSS: 1.2 M KCl, 3 mM NaN₃, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 4 mM NaHCO₃, 2 mM EGTA at pH 7.0] and 50% glycerol at -20°C.

The syphon muscle was dissected live from the squid, *L. pealeii*, and homogenized at 4°C in two volumes of half strength motility buffer (1/2×) (Vale *et al.*, 1985) containing protease inhibitors (Bearer *et al.*, 1993). The homogenate was microfuged at 8,000×g for 5 min, washed twice in 1/2×, and the pellet resuspended in 0.6 M KI, 4 mM MgCl₂, 4 mM EGTA, 10 mM DTT and 20 mM imidazole-HCl, pH 7.0 and microfuged. The myosin-rich supernatant was either further extracted with ammonium sulfate for electronmicroscopy or precipitated for SDS-gels. In the latter case, myosin was diluted in 15 volumes of cold water, and after 1 h on ice, it was collected by microfuging at 16,000×g for 20 min and resuspended in MSS and 50% glycerol for storage at -20°C. Purification was determined by Coomassie-stained SDS-gels.

Further separation of myosin II from paramyosin, actin and other minor contaminants was achieved by selective ammonium sulfate precipitation. A concentration of 10 mM MgCl₂, 10 mM ATP, and a final saturation of 35% ammonium sulfate was added to the previous myosin-rich supernatant and incubated for 5 min on ice. Contaminants were microfuged at 16,000×g for 10 min and the supernatant was dialyzed into 0.5 M KCl, 3 mM NaN₃, 2 mM MgCl₂, 1 mM DTT, 4 mM NaHCO₃, 2 mM EGTA at pH 7.0 overnight. An equal volume of glycerol was added to the dialyzed myosin and stored at -20°C.

Electron microscopy of myosin molecules

Squid myosin II, further purified by ammonium sulfate precipitation, at a concentration of 100 µg/ml, was sprayed onto mica and shadowed with platinum/carbon at 11° (Tyler and Branton, 1980; Bearer *et al.*, 1996a). Replicas were examined in JEOL 200CX electron microscope.

Antibody production

Syphon muscle myosin II was further purified for antibody production by SDS-PAGE. The ~210-kDa band was excised from the gel and sent to ICN Biomedicals, Inc. (Costa Mesa, CA) for commercial antibody production, where it was emulsified and mixed with phosphate-buffered saline for a final volume of 12 ml. Three aliquots of 4 ml each were mixed with an equal volume of Freund's adjuvant and injected subcutaneously into multiple sites on the neck and back of the rabbit. Serum, obtained at one month intervals, was titrated on purified myosin II by Western blot.

Western blots

Western blots were performed as previously described (Bearer *et al.*, 1993) except anti-myosin antibodies were used at 1:500 dilution and the secondary alkaline phosphatase-conjugated antibodies at 1:5000 (Boehringer Mannheim, Indianapolis, IN) and developed with BCIP/NBT phosphate substrate (Kirkgaard and Perry Laboratories, Gaithersburg, MD).

Amino acid sequencing

Squid syphon muscle myosin II (40 µg) was pelleted out of MSS after dilution in 10 volumes of NED (0.1 mM NaHCO₃, 0.1 mM EGTA and 0.1 mM DTT) and dissolved in 80 µl 0.1% SDS/100 mM NH₄HCO₃ (digestion buffer, DB), and heated at 95–100°C for 5 min (Riviere *et al.*, 1991). The resulting denatured protein solution was applied to a DB-equilibrated Biospin 30 chromatography column (Bio-Rad Laboratories, Hercules, CA) and processed according to the manufacturer's protocol. The resulting >40-kDa fraction was either digested at 37°C overnight with 1.5 µg endoproteinase Lys-C (Wako Chemicals USA Inc., Richmond, VA) in 6 µl of DB, or subjected to reduction and alkylation by addition of 5 µl of 45 mM DTT at 50°C for 15 min followed by addition of 5 µl of 100 mM iodoacetamide for 15 min at room temperature (Stone *et al.*, 1990) and then digested. The resulting digests were adjusted to a volume of 150 µl with DB and 150 µl of 1 M guanidinium hydrochloride which precipitated the SDS (Riviere *et al.*, 1991).

The precipitate was removed by centrifugation and then the supernatant was passed through a Millex HV filter unit (Millipore Corporation, Bedford, MA) prior to loading on a reversed-phase high performance liquid chromatography column (RP-HPLC) (Step 1) with a narrow-bore (2.1× 250 mm) Vydac 218TP52 column and guard column (Separations Industries, Metuchen, NJ), eluted at 0.25 ml/min at 35°C utilizing a gradient (Fernandez *et al.*, 1992) on a System Gold HPLC equipped with a Model 507 autosampler, Model 126 programmable solvent module and Model 168 diode array detector (Beckman Instruments, Inc., Fullerton, CA). Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA

in acetonitrile. Fractions were collected at 30-s intervals and stored at -70°C . Selected fractions from the digest of non-reduced and alkylated myosin that eluted at least several minutes apart were pooled and concentrated in a Savant Speed Vac concentrator and re-chromatographed (Step 2) on a narrow bore (2.0×250 mm) YMC ODS AQ column (YMC Inc., Wilmington, NC) eluted at 0.25 ml/min at 35°C . Column eluate was monitored at 215 and 280 nm. Fractions were collected at 30-s intervals and stored at -70°C .

Fractions (125 μl) containing peptides purified by one or two RP-HPLC steps were applied in 30- μl aliquots to a Biobrene-treated glass fiber filter (Applied Biosystems, Foster City, CA) and dried prior to amino acid sequencing on a Model 477A pulsed-liquid protein sequencer equipped with a Model 120A PTH analyzer (Applied Biosystems) using methods and cycles supplied by the manufacturer. Data was collected and analyzed on a Model 610A data analysis system (Applied Biosystems). Amino acid sequences were searched by *fasta* in the GCG-Swiss Protein Database (University of Wisconsin Genetics Computer Group). Sequences were matched using the *gap* program.

Preparation of squid optic lobe myosin

Squid brain myosin was extracted according to See and Metuzals (1976). The 75% saturated ammonium sulfate precipitate containing the myosin was resuspended in 3–5 ml of MSS and an equal volume of glycerol, yielding approximately 2 mg/ml of protein, and stored at -20°C overnight. Myosin was precipitated out of glycerol by dilution into 10 volumes of cold NED and stirred for 10 min at 4°C . Myosin was pelleted out of the mixture by centrifugation at $5000\times g$ for 30 min and resuspended in 0.5 ml 50 mM sodium pyrophosphate, 100 mM KCl, 0.5 M sucrose, 20 mM Tris, pH 7.5, 4 mM MgCl_2 , 1 mM EGTA, 5 mM DTT and 0.5 mM MgATP. Finally, solubilized myosin was centrifuged at $50,000\times g$ for 30 min. Squid optic lobe myosin is present in the supernatant.

Sequencing of squid optic lobe myosin

Semi-pure squid optic lobe myosin was gel-purified and the ~220-kDa myosin was excised and subjected to *in situ* proteolytic digestion with endoproteinase Lys-C in the presence of 0.1% SDS according to Kawasaki *et al.* (1990). The resulting digest was subjected to purification by two RP-HPLC steps on Vydac 218TP52 (Separations Industries) and YMC ODS AQ columns (YMC Inc.) after prior removal of the SDS by precipitation with guanidinium hydrochloride and filtration with a Millex HV filter (Millipore Corporation). Resultant pure peptides were subjected to Edman degradation, outlined above, yielding 11 amino acid sequences of 9–25 residues long from this ~220 kDa squid optic lobe myosin.

Creating a myosin directory

Myosin sequences (125) were retrieved from the Genbank and Swiss Protein data banks and assembled into a directory. The directory contained at least two myosins from each of the classes I, II, III, IV, V, VI, VII, VIII and IX. The sequences in the directory are listed by accession numbers: A23662, A23695, A26045, A32491, A33620, A33977, A35082, A35557, A36014, A43298, A45438, A45439, A45627, A47297, A48467, A49354, A53933, A54818, A60877, A61050, B32491, B43402, B45439, C45439, M74066, P02562, P02563, P02564, P02565, P02566, P02567, P04460, P04461, P04462, P05659, P05661, P06198, P08799, P08964, P10567, P10568, P10569, P10587, P10676, P10677, P11055, P11778, P12844, P12845, P12847, P12882, P12883, P13392, P13533, P13535, P13538, P13539, P13540, P13541, P14105, P19524, P19706, P22467, P24733, P29616, P32492, P34092, P34109, P35415, P35416, P35417, P35418, P35579, P35580, P35748, P35749, P39922, Q01989, Q02171, Q02440, Q03479, Q05000, Q05870, Q99104, Q99105, Q99323, S03166, S05806, S06812, S12323, S16600, S16601, S16602, S19188, S29984, S31926, S33812, S35628.pir2, S38572, S39214, S41749, S46444, S47106, S47107, S49119, S49153, S49478,

U03420, U04049, U14370, U14371, U14372, U14373, U14374, U14375, U14376, U14377, U14378, U14379, U14380, U14381, U14391, U14549, and U17180. This directory was used as a file upon which to apply the *GCG* search command *fasta*.

Purifying squid axoplasmic organelles

Axoplasm was extruded from the giant axon of *Loligo pealeii* and organelles washed in buffered 0.6 M KI and separated from cytoplasmic and cytoskeletal components through a sucrose density gradient (Bearer *et al.*, 1993, 1996b). Organelles focus in the 15% sucrose step, while actin, tubulin, neurofilaments are solubilized and excluded from the gradient.

RESULTS

Squid syphon muscle myosin II

Myosin II from the syphon muscle of the squid, *Loligo pealeii*, is enriched with substantial yield by cycling the extracted muscle proteins through a series of centrifugations in high or low salt buffers (Fig. 2A). This approach yields a prominent band (Fig. 2B, lane 1) migrating slightly higher than purified rabbit muscle myosin II (200 kDa; Fig. 2B, lane 2), as well as 43-kDa and 110-kDa bands, presumably actin and paramyosin, respectively. This prominent band migrated at ~210 kDa, though precise molecular weights of proteins migrating slower than 200 kDa is difficult to assign. Purification from the mantle muscle using a previously published protocol (Konno, 1978) produced lower yields of the ~210-kDa band and failed to separate it from two other higher molecular weight bands (Fig. 2B, lane 3). This is probably because, unlike syphon muscle, mantle contains large amounts of collagen and other large connective tissue proteins. Application of this protocol to a species different from *Ommastrephes sloani pacificus*, for which it was originally designed, could also account for the yields and purity being less than those achieved using our new protocol where syphon muscle is the starting material.

To confirm that this high molecular weight band was indeed myosin, we examined it by glycerol spray electronmicroscopy. To prepare the protein for glycerol spray we precipitated the paramyosin and actin with 35% ammonium sulfate (Fig. 3, lanes A–C). After dialysis, the supernatant (Fig. 3, lanes C, D and E) was significantly depleted of paramyosin and actin, and contained the prominent ~210-kDa band as well as four other, unidentified, bands of lesser intensity migrating between 66 and 34 kDa. In 15% gels, both light chains were clearly visible at ~18 and ~20 kDa (Fig. 3, lane E). By densitometry there was at least one of each light chain per heavy chain.

In rotary shadowed replicas (Fig. 4), individual molecules of squid muscle myosin II frequently appear with the familiar two-heads and sinuous tail typical of vertebrate myosins (Elliott *et al.*, 1976; Shotten *et al.*, 1979). The tails of the squid myosin II are similar in length (150 nm) to that of rabbit myosin. Also present are multiple circular forms (Fig. 4, bottom row) of circumference equal to the length of the myosin tail, each with a single knot representing one, or possibly two myosin heads. The thickness of the rest of the ring was the same as that of the tail of the two-headed linear molecule. The numbers of circular forms varied in different preparations and in different areas of a replica from a single preparation—in some areas up to 30% of the molecules were in circular forms. This number of circular forms in preparations containing a concentration of 0.15 g/L myosin II gives a K_a of $\sim 10^{-9}$ M⁻¹ head–tail binding, where $K_a = [\text{circular forms}] / ([\text{free heads}][\text{free tails}])$.

To obtain the amino acid sequence from the squid muscle myosin, 40 µg of the pellet resulting from the last step of the squid muscle enrichment procedure (Fig. 2A, lane 6) was digested by limited proteolysis, and the peptide fragments separated by HPLC (Fig. 5A). Reduction of the protein prior to digestion gave essentially the same elution profile as non-

reduced protein (Fig. 5B). In-gel digestion of the ~210-kDa band gave essentially the same elution pattern. Peptides present in peaks were further separated by a second HPLC fractionation (Fig. 5C). Twenty-two peaks contained sufficient amounts of protein to be sequenced by Edman degradation, and three of these were overlapping sequences. Sequences ranged from 8 to 30 amino acids long.

These amino acid sequences were first screened by a *fasta* search against the GenBank database (Devereux *et al.*, 1984), and then each of the sequences matching myosins were compared to scallop and chick pectoralis muscle myosin II (Fig. 6 and Table 1). Amino acid sequencing demonstrated that myosin, paramyosin and actin are present in this pellet (Table 1), and all amino acid sequences matched one or the other of these proteins with high homologies. No light chain sequences were recovered. Fourteen of the sequences matched myosins, two of these were in the motor domain; one matched paramyosin, and four matched actin. All sequences matched scallop myosin better than chick, with similarities ranging from 75 to 100%, and identities from 50 to 95%. Surprisingly, the highest degree of identity between squid and scallop myosins, on the basis of sequencing peptide fragments, is in the tail, at amino acid positions (*aa*) 1504–1518 (87%) and *aa*1535–1554 (95%) of scallop myosin. All sequences also matched chick pectoralis muscle myosin II but with slightly lower percentage identities, ranging from 31 to 83%. Again, the highest amino acid identity between squid and chick myosin occurred in the tail, also corresponding to scallop *aa*1504–1518 (83%) and *aa*1535–1554 (80%; Table 1). A map showing where these sequences match the complete sequence of scallop muscle demonstrates that only two arise from the head domain, while 12 match the tail (Fig. 6).

Optic lobe myosin

In order to identify a nervous system myosin, we chose to prepare high molecular weight myosins from squid optic lobe. To select for such myosins, we raised antibodies against gel-purified squid muscle myosin II and used them as a probe for myosins in optic lobe. Polyclonal antibodies raised against the gel-purified squid muscle myosin II band were first characterized in Western blots of squid muscle homogenates where they recognized a single ~210-kDa band (Fig. 7, lanes A and B). These antibodies also recognized an ~220-kDa band in homogenates of squid optic lobe (Fig. 7, lanes C and D). A modification of a protocol for the purification of a putative myosin II from squid optic lobe (See and Metuzals, 1976) produced a preparation enriched in the ~220-kDa band (Fig. 7, lane E).

This broad protein (stained by Coomassie) band, migrating from 210–220 kDa, was separated from contaminating proteins by gel electrophoresis and excised from the gel for sequencing. Digestion of 20 µg of this optic lobe protein band in the presence of SDS produced numerous fragments which were separated by two successive reverse phase HPLC fractionations. This yielded eleven peptides of sufficient quantity and purity for Edman degradative sequencing.

The resulting peptide sequences were matched by *fasta* to the protein sequences in Swiss Protein Databank. Myosin II sequences were present in the top ten *fasta* matches for six of the peptides (Table 2A). Comparison to scallop myosin II was then performed by a gap sequence alignment. The remaining five peptides matched myosin II with high densities (50–60%) and four of these also matched unconventional myosins (33–50% identities) (Table 2B). These five sequences also matched numerous other proteins that were not myosin, making them less useful for identification. Four sequences of the eleven were found to match in the head, and seven in the tail domain.

Many peptides, particularly those five that matched with lower percentage identities, matched to unconventional myosins in the same region as to myosin II, including myosin I,

V and VI. The first unconventional myosin found by a *fasta* search of our myosin directory (see Materials and Methods for a list of the accession numbers of sequences included in this directory), is listed below the top *fasta* match of our directory (Table 2B). These five sequences were also aligned with scallop muscle myosin.

All 11 sequences matched some other non-muscle myosin at least as well or better than they matched the scallop muscle myosin. Many matched to an unconventional myosin better than to scallop muscle myosin II, even though this myosin is from a related species. The most common match was to non-muscle/smooth muscle myosin IIs, suggesting that this is the only or at least the predominant myosin class in the myosin-enriched band from which the sequences were obtained.

Myosin copurifying with axoplasmic organelles

Finally, the squid muscle myosin II antibodies were used to probe Western blots of organelles isolated from axoplasm by centrifugation through a sucrose step gradient (Bearer *et al.*, 1993). The anti-squid muscle myosin II antibodies recognized a single band which typically migrated at ~220 kDa (Fig. 8).

DISCUSSION

Here we report a strategy which resulted in the identification of an axoplasmic myosin associated with motile organelles. This strategy was designed to overcome the difficult problem of identifying a myosin associated with axoplasmic organelles that we had originally detected using cross-reactive anti-myosin antibodies raised against a muscle myosin from another species (Bearer *et al.*, 1993). Other more direct approaches to define this myosin have failed because not enough protein could be obtained, even from the giant axon of the squid, to permit amino acid sequencing or the generation of antibodies.

The strategy reported here was suggested by that used to isolate a myosin from *Tetrahymena* (Garcés *et al.*, 1995), another situation where biochemical purification of sufficient protein was not readily performed. The strategy depends on using squid muscle myosin as a source of protein for developing peptide sequencing protocols and for generating anti-myosin antibodies. Since the subclass of myosin(s) copurifying with organelles is not known, we adopted this approach because it had the potential of identifying any myosin regardless of subtype. We reasoned that antibodies against the full-length squid muscle myosin II would be likely to include those recognizing conserved regions common to many or all myosin subclasses, and thus be likely to detect any myosin regardless of its class.

The squid muscle myosin II also provided us with an abundant source of protein for the selection of a peptide sequencing protocol applicable to small amounts of myosin. In the process of developing such a protocol, we obtained a number of peptide sequences for this myosin that proved useful in cloning and sequencing it (Matulef *et al.*, 1998). Comparison of these peptide sequences with other myosins reveal clues about tail structure and function, even though only 257 residues, representing 13% of the protein, were obtained.

Syphon muscle myosin II has a high degree of identity with myosin II from scallop and chick. The high degree of conservation in an amino acid domain of the tail (aa1504–1518) and (aa1535–1554) suggests that this domain serves a significant functional role, possibly in regulating thick filament formation in all three organisms (McLachlan and Karn, 1982). It would potentially be interesting to compare more of the sequence of paramyosins from *Taenia* and *Loligo*, since the myosin tails in these species must interact with paramyosins, while the chick myosin does not require paramyosin to form thick filaments.

In the process of exploiting muscle myosin to identify the organelle motor, we gained insight into other details about squid muscle myosin: a purification protocol was devised; antibodies generated; and structural studies revealed head–tail interactions. Since our goal was not to investigate muscle myosin, we have not pursued these observations further. However, it would be interesting to discover the biochemical basis of the circular forms as well as their physiologic function, if any. Such structures have not been imaged before, despite biochemical evidence that other myosin IIs adopt this conformation (Cross *et al.*, 1988). Furthermore, the relatively low K_a we calculate strongly supports a physiological role. The presence of stoichiometric amounts of light chains and the circular rather than clover-leaf structures observed demonstrate that this conformation is unlikely to be a result of hydrophobic interactions between neck and tail (Trybus and Lowey, 1988). It is tempting to postulate a functional role for head–tail binding since many other myosins head–tail interactions regulate thick filament formation, (Citi *et al.*, 1987; Cross *et al.*, 1988), actin binding, and *in vitro* motility (Kuczmarski and Spudich, 1980; Korn, 1982; Lee *et al.*, 1994; Olney *et al.*, 1996; Shoffner and De Lozanne, 1996).

The anti-muscle myosin antibodies detected a single band in squid optic lobe homogenates of ~220 kDa. Partial purification of this band and subsequent peptide sequencing reveal it to be a myosin II, possibly of the myosin IIB subclass. That it is a myosin II is not surprising, since the antibodies were raised against a myosin II and the purification strategy we followed was a modification of a protocol devised to isolate myosin II from squid brain long before the other subclasses were recognized (See and Metzuzals, 1976). The peptide sequences match other myosins of the IIB subclass, so called because it is the predominant cytoplasmic myosin isoform found in brain. However, whether this is the squid counterpart of myosin IIB must await the identification of all the myosin IIs from squid and comparison of their sequences.

Affinity-purified antibodies of squid muscle myosin probing Western blots of isolated axoplasmic organelles recognized a single band of similar mobility as that detected in optic lobe. Cosedimentation of this myosin with KI-washed organelles suggests it has a strong and specific interaction with them, especially since 0.6 M KI is a strong chaotropic agent known to strip dynein but not kinesin off of organelles (Schnapp *et al.*, 1992). Furthermore, the KI wash also solubilizes cytoskeletal components and has long been known to dissociate myosin thick filaments and myosin-actin interactions. It is therefore unlikely that this myosin sediments in the 15% fraction independent of the organelles. Organelles isolated from axoplasm according to this protocol are motile on either microtubules (Schnapp *et al.*, 1992) or actin (Bearer *et al.*, 1996b). Thus, the presence of the myosin in the 15% sucrose gradient fraction containing the organelles strongly supports both its specific association with these organelles and its potential role as an actin-based motor mediating their movements within the neuron.

In the absence of sequence from the organelle myosin, it is not possible to be absolutely certain that the band recognized by the antibodies in the organelle preparation is the same as that recognized in the optic lobe preparation, from which our 11 peptide sequences were derived. However, the antibodies recognize only one band in the organelle preparation, and this band is the same apparent molecular weight as the protein isolated from optic lobe. Thus, by all criteria available at this time, the two proteins appear the same.

Could a myosin II, thought to be a contractile, bipolar, filamentous myosin, be present on organelles? The myosin isolated from axoplasm does not appear to form bipolar filaments even at low salt, though it can self-associate with several heads all at one end (Bearer *et al.*, 1996a). A myosin IIB identified in rat brain has also been implicated in membrane binding (Li *et al.*, 1994). Differences in ultrastructure within most of the myosin subclasses have not

yet been defined, but it is not unreasonable to suggest that the coil–coil domain of the myosin tail which mediates thick filament formation in muscle might make unipolar complexes in axoplasm, the tails of which could, in turn, associate with organelles. In any instance, the present approach has allowed us to develop tools, obtain peptide sequences, and generate antibodies that indicate that at least one of the axoplasmic organelle myosins is a myosin II.

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Fig. 1.

Strategy to identify an organelle-associated myosin. Myosin is first purified from squid syphon muscle and its identity confirmed by EM. This muscle myosin is then used: (1) to generate anti-myosin antibodies; and (2) to optimize peptide-sequencing protocols. Resultant myosin antibodies are used to probe Western blots of squid optic lobe and axoplasmic organelles. The myosin detected by these antibodies is then purified from optic lobe and peptide sequences obtained. The myosin antibodies are then used to determine whether the organelle fraction contains a protein of the same size as the one in optic lobe, which is also recognized by these myosin antibodies.

Fig. 2.

Comparison of muscle myosin enrichments on 8.5% SDS-gels. (A) Enrichment of myosin II from squid syphon muscle. Lane 1: homogenate; lanes 2–3: low salt supernatant; lane 4: high salt supernatant; lane 5: high salt pellet; and lane 6: final step with enrichment of a ~210-kDa protein. In addition to this band, the final preparation contains a 43-kDa and a 110-kDa species (actin and paramyosin) which were also abundant in the starting material. Dashes: 200, 116, 45, and 34 kDa. (B) Comparison of the syphon myosin with rabbit muscle and squid mantle myosins. Lane 1: syphon after ammonium sulfate extraction; lane 2: rabbit; lane 3: mantle. Note that syphon myosin migrates slightly slower than rabbit myosin II and that protocols applied to syphon produce fewer additional bands than when applied to mantle.

Fig. 3. Ammonium sulfate precipitation removes the majority of paramyosin and actin from the preparation. Lane A: final step of myosin enrichment. Lane B: ammonium sulfate pellet. Lane C: ammonium sulfate supernatant. Arrow points to ~210 kDa myosin that was retained. Arrowheads point to paramyosin (~110 kDa) and actin (~42 kDa) that were precipitated. Dashes: 200, 116, 98, 66 and 45 kDa. Lane D: the ammonium sulfate supernatant after dialysis separated on 8.5% Coomassie-stained SDS-gel. Arrow points to myosin. Dashes: 116, 98, 66 and 45 kDa. Lane E: ammonium sulfate supernatant electrophoresed on 15% SDS-gel to reveal the presence of bands representing light chains (arrows). Dashes: 200, 98, 66, 45, 31, 22 and 15 kDa.

Fig. 4. Molecular structure of myosin II. The first seven panels show low angle rotary shadowed squid syphon muscle myosin II. Rabbit muscle myosin shows a similar but not identical morphology. Bottom row shows ring structures present in up to 30% of all molecules observed in preparations of syphon muscle myosin II. Bar=50 nm.

Fig. 5. HPLC elution profiles of myosin peptides. (A) Step 1 of the endoproteinase Lys-C digestion of 40 μ g of reduced and alkylated squid myosin. (B) Step 1 of the endoproteinase Lys-C digestion of 40 μ g of untreated squid myosin. (C) Step 2 of the pooled fractions eluting at 37.0, 45.0, 47.0, 52.5, 58.0, 68.5, 70.5, and 75.0 min (see B above) from the endoproteinase Lys-C digestion of 40 μ g of squid myosin.

HEAD	NECK	TAIL					
$\overline{271}$ 85%		$\overline{923}$ 60%	$\overline{1060}$ 85%	$\overline{1250}$ 50%	$\overline{1504}$ 87%	$\overline{1700}$ 71%	$\overline{1857}$ 67%
$\overline{301}$ 66%		$\overline{975}$ 67%	$\overline{1097}$ 50%	$\overline{1277}$ 69%	$\overline{1535}$ 95%	$\overline{1756}$ 76%	$\overline{1898}$ 60%

Fig. 6.

Squid myosin II peptides mapped on a diagram of scallop myosin II. Limited digestion of syphon myosin II produced two peptide sequences that matched to the head and 12 that matched along the tail domain of scallop myosin II. Each bar indicates the approximate position on the diagram of scallop myosin II where each peptide shows highest identity. Below the bar is shown the number of the first amino acid position of the scallop myosin. Below the *aa* position is shown the percentage identity between peptide and scallop myosin II. The peptides are mapped to scale, but the length of the bar is arbitrary.

Fig. 7.

Squid myosin II polyclonal antibodies recognize myosin in optic lobe. Lane A: squid syphon muscle homogenate on 8.5% SDS-gel. Arrowhead points to ~220-kDa myosin. Lane B: parallel western blot with 10-fold less protein loaded than lane A probed with squid myosin II antibodies. Lane C: squid optic lobe homogenate. Arrowhead points to ~220-kDa band presumably containing myosin. Lane D: corresponding Western blot probed with squid muscle myosin II antibodies. Dashes: 200, 116, 98 and 66 kDa. Lane e: preparation of squid optic lobe myosins on 8.5% SDS-gel. Arrowhead indicates the band that was excised and subjected to proteolysis and sequencing. Dashes: 200, 116, 98 and 66 kDa.

Fig. 8. Myosin co-purifies with squid axoplasmic organelles. Gel: 8.5% SDS-gel of axoplasmic organelles purified by sucrose density gradient. Blot: corresponding Western blot with affinity purified anti-squid myosin II antibodies. Arrowhead indicates the ~220-kDa myosin. Dashes: 200, 116, 98, 66 and 45 kDa.

Table 1

Comparison of sequences from squid syphon muscle with scallop and chick pectoralis myosins

Organism	Sequence	Amino acid position	Similarity (per cent)	Identity (per cent)
Myosin				
Squid (#10)	XRVTYQQAERNYHIFYQLLS			
Scallop	SRVTYQQAERNYHIFYQICS	271–291	90	85
Chick	SRVTFQLPAERSYHIFYQIMS	273–294	80	66
Squid (#7=1)	ILAVDPGLYGFINGTGLTVDGIDDXEEMG			
Scallop	MLVTPDSGLYSFINQGCLTVDNIDDVVEEFK	301–330	76	66
Chick	LLITTNPYDYHYVSQGEITVPSIDDQEELM	303–332	63	40
Squid (#15)	LMDEEDAATELSAQK			
Scallop	LLDEEDAAADLEGIK	923–937	80	60
Chick	AEDEEEINAELTAKK	928–942	53	46
Squid (#19)	TLQDEMAQQDEHLSK			
Scallop	TLQGEISQQDEHIGK	975–989	93	67
Chick	NLTEEMAVLDETIK	978–992	80	47
Squid (#18)	TTQETVEDLERVK			
Scallop	STQENVEDLERVK	1060–1072	85	85
Chick	LAHDSIMDLENDK	1063–1075	53	31
Squid (#20)	VVSEWQHK			
Scallop	LVSQQLRK	1097–1103	100	50
Chick	EISQIQSK	1088–1095	62	38
Squid (#16)	EAQLSEXNAK			
Scallop	EAQLSEXNAK	1250–1259	80	50
Chick	EDQLSEIKTK	1255–1264	60	60
Squid (#6–11)	SRLQTEADLTRQLEEAHNVGQLTK			
Scallop	SRLQAENSDLTRQLEDAEHRVSVLSK	1277–1302	76	69
Chick	ARLQTETGEYSRQAEKDALISQLSR	1279–1304	65	42
Squid (#14)	LADEIHDXTDQLGEG			
Scallop	LADEIHDLTDLQSEG	1504–1518	93	87
Chick	LQQEIADLTEQIAEG	1507–1521	80	83
Squid (#21)	EELQAALAEAAALEQEEAK			
Scallop	EELQAALAEAAALEQEEAK	1535–1554	100	95
Chick	SELQAALAEAAALEHEEGK	1540–1559	90	80
Squid (#17)	AAENELADASDRVNELQAQVSTVG			
Scallop	ASDNELADANDRVNELTSQVSSVQ	1700–1723	75	71
Chick	VAEQELLDATEVQLLHTQNTSLI	1703–1726	54	42
Squid (#22)	HAMADATRLADELRQEVDHGLSVEK			
Scallop	KAMADAARLADELRAEQDHSNQVEK	1756–1780	80	76
Chick	KAITDAAMMAEELKKEQDTSAHLE	1759–1783	68	40
Squid (#13)	NYERMQELVDKLQNK			
Scallop	NQERLQELIDKLNAK	1857–1871	80	67

Organism	Sequence	Amino acid position	Similarity (per cent)	Identity (per cent)
Chick	NILRLQDLVDKQLQMK	1872–1886	80	67
Squid (#9–12)	VQQELEDAEERADQSEGALQ			
Scallop	AQHELEEAERADTADSTLQ	1898–1917	80	60
Chick	IQHELEEAERADIAESQVN	1903–1922	80	55
Paramyosin				
Squid (#8)	KYETDIRELESALDTANRQNAE			
Scallop myosin	KLEQDINELEVALDASNRGKAE	1616–1637	63	63
Paramyosin	KYEATISELEVQLDVANKANAS	531–552	62	59
Actin				
Squid	AGFAGDDAPRAVFPISIVGRPRHQGVMMGMGQK			
<i>C. elegans</i>	AGFAGDDAPRAVFPISIVGRPRHQGVMMGMGQK	20–51	100	100
Squid	YPIEXGIVTNWDDMEK			
<i>C. elegans</i>	YPIEHGIVTNWDDMEK	71–86	100	94
Squid	IWHHTFYNELRVAPEEHPVL			
<i>C. elegans</i>	IWHHTFYNELRVAPEEHPVL	87–106	100	100
Squid	EITSLAPSTMK			
<i>C. elegans</i>	EITALAPSTMK	316–326	90	90

Sequences with high homology to myosin II, actin and paramyosin were obtained from the digest of the low salt pellet from the final step in purification. The figure shows the line-ups and homologies. Scallop myosin sequence (P24733): Nyitray *et al.*, 1991; chick pectoralis muscle myosin sequence (P13538): Maita *et al.*, 1991 and 1987; *Caenorhabditis elegans* sequence (P10983): Krause *et al.*, 1989; *Taenia soleum* paramyosin sequence (P35418): Landa *et al.*, 1993. Numbers in parentheses are reference numbers for our internal data bank.

Table 2A*Fastas* of the peptide sequence obtained from the ~220-kDa band from squid optic lobe

Organism	Sequence	Amino acid position	Similarity (per cent)	Identity (per cent)
Myosin				
Squid optic lobe (#3)	KGDEVVVDVEDTGK			
Myosin II, smooth muscle [rabbit]	KGDEVTVELQENGK	53–66	79	64
Myosin II, muscle [scallop]	KGDEITVKIVADSS	52–65	50	36
Squid optic lobe (#6)	KRTTFHRDDIQK			
Myosin II, smooth muscle [chicken]	KKVTLSKDDIQK	66–77	75	58
Myosin II, muscle [scallop]	STRTVKKDDIQS	65–76	50	42
Squid optic lobe (#9)	KVGRDHVTK			
Myosin IIA, nonmuscle [chicken]	KVGRDYVQK	397–405	89	78
Myosin II, muscle [scallop]	KVGTEMVTK	404–412	78	67
Squid optic lobe (#8)	KXAQVEEVQSQLARREEELQSALQK			
Myosin II, smooth muscle [chicken]	LQAQIAELKAQLAKKEELQAALAR	1079–1103	68	52
Myosin I [bovine]	DQEGVEKVLGELSMSSEELAFGKTK	653–677	36	32
Myosin II, muscle [scallop]	KLEDEQNLVSQLQRKIKELQARIEE	1091–1115	48	32
Squid optic lobe (#4)	XLELRISELEDLLDEEQ			
Myosin IIA, nonmuscle [chicken]	RLEARIAQLEEELEEEQ	1726–1742	76	59
Myosin II, muscle [scallop]	KLESRVHELEAELDNEQ	1811–1827	65	59
Squid optic lobe (#11)	KMQTEYEQAV			
Myosin II, cardiac muscle [rat]	QLQTEVEEAV	1736–1745	80	60
Myosin II, muscle [scallop]*	AMQTDLDEMh	1733–1742	50	40

For these six sequences, several myosins appeared among the first ten matches. Shown is the top myosin match as well as comparison to sequence from scallop myosin II, a close relative. Scallop myosin sequence (P24733): Nyitray *et al.*, 1991.

Table 2B

Squid optic lobe fragments are compared to a databank created in our lab (see Materials and Methods) of representative myosin sequences from all classes and to scallop myosin II

Organism	Sequence	Amino acid position	Similarity (per cent)	Identity (per cent)
Myosin				
Squid optic lobe (#5)	KLLGHHVNHPK			
Myosin IIB, nonmuscle [fruit fly]	KLVSASHMHPK	601–611	55	55
Myosin VI [fruit fly]	TLLDEESKLPK	528–538	36	36
Myosin II, muscle [scallop]	KLYQNHMGKNR	550–560	36	27
Squid optic lobe (#1)	KXELEVNDLK			
Myosin II, heavy chain D [<i>C. elegans</i>]	KAQQEVENLK	949–958	80	60
Myosin V [chicken]	GAELEYESLK	1203–1212	70	50
Myosin II, muscle [scallop]	KKKMEADNAN	937–946	50	40
Squid optic lobe (#2)	FEYEDLERK			
Myosin II, skeletal muscle [rat]	FEYNSLEQL	1086–1094	67	56
Myosin VI [pig]	FEYFEHNSF	1149–1157	33	33
Myosin II, muscle [scallop] *	ENVEDLERV	1061–1069	56	56
Squid optic lobe (#7)	KQGENQALELRQK			
Myosin II, heavy chain D [<i>C. elegans</i>]	KQYEIQVAELQQK	1257–1269	62	62
Myosin VI [pig]	KQEEEEAERLRRI	918–930	54	46
Myosin II, muscle [scallop]	KQFESQMSDLNAR	1247–1259	54	38
Squid optic lobe (#10)	KVSNLQTDLA			
Myosin II, cardiac muscle [human]	KVEKLRSCLS	1136–1145	50	50
Myosin II, muscle [scallop]	KIKELQARIE	1104–1113	50	30

* Indicates that the match between the peptide sequence and the scallop muscle myosin was 'forced' by restricting comparison to the domain of the sequence that *fasta* picked for the top myosin match. Numbers in parentheses are reference numbers for our internal data bank.

Bovine myosin I sequence (P10568): Hoshimaru *et al.*, 1987; *C. elegans* muscle myosin II sequence (P02567): Dobb *et al.*, 1989; chicken myosin V sequence (S19188): Espreafico *et al.*, 1992; chicken nonmuscle myosin IIA sequence (P14105): Shohet *et al.*, 1989; chicken smooth muscle myosin II sequence (P10587): Yanagisawa *et al.*, 1987; fruit fly myosin VI sequence (Q01989): Kellerman *et al.*, 1992; fruit fly nonmuscle myosin IIB sequence (A36014): Ketchum *et al.*, 1990; human cardiac muscle myosin II sequence (P13533): Matsuoka *et al.*, 1991; pig myosin VI sequence (A54818): Hasson and Mooseker, 1994; rabbit smooth muscle myosin II sequence (P35748): Babij *et al.*, 1991; rat cardiac muscle myosin II sequence (P02564): Kraft *et al.*, 1989; rat skeletal muscle myosin II sequence (P12847): Strehler *et al.*, 1986; scallop myosin II sequence (P24733): Nyitray *et al.*, 1991.