Association of a Non-muscle Myosin II with Axoplasmic Organelles

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ABSTRACT

Association of motor proteins with organelles is required for the motors to mediate transport. Since axoplasmic organelles move on actin filaments, they must have associated actin-based motors, most likely members of the myosin superfamily. To gain a better understanding of the roles of myosins in the axon we used the giant axon of the squid, a powerful model for studies of axonal physiology. First, a ~220 kDa protein was purified from squid optic lobe, using a biochemical protocol designed to isolate myosins. Peptide sequence analysis, followed by cloning and sequencing of the full-length cDNA, identified this ~220 kDa protein as a non-muscle myosin II. This myosin is also present in axoplasm as determined by two independent criteria. First, RT-PCR using sequence-specific primers detected the transcript in the stellate ganglion, which contains the cell bodies that give rise to the giant axon. Second, Western blot analysis using non-muscle myosin II isotype-specific antibodies detected a single ~220 kDa band in axoplasm. Axoplasm was fractionated through a four-step sucrose gradient after 0.6 M KI treatment which separates organelles from cytoskeletal components. Of the total non-muscle myosin II in axoplasm 43.2% co-purified with organelles in the 15% sucrose fraction, while the remainder (56.8%) was soluble and found in the supernatant. This myosin decorates the surfaces of 21% of the axoplasmic organelles, as demonstrated by immunogold electron-microscopy. Thus, non-muscle myosin II is synthesized in the cell bodies of the giant axon, is present in the axon, and is associated with isolated axoplasmic organelles. This myosin therefore is likely to be, in addition to myosin V, an axoplasmic organelle motor.

Key words: Squid non-muscle myosin II, Actin-based organelle transport, Fast axonal transport
INTRODUCTION

Biochemical association between organelles and the microtubule motors, kinesin and cytoplasmic dynein, has provided some of the more convincing evidence that these motors play a role in intracellular transport (Schnapp and Reese, 1989; Schnapp et al., 1992; Yamazaki et al., 1995; Moreira et al., 1998). Strong evidence for a physiological association of kinesin with organelles was obtained by the retention of kinesin on organelles after extraction with potassium iodide (Schnapp et al., 1992). Immunogold labeling has been used to link specific motors with organelles (Yamazaki et al., 1995; Moreira et al., 1998).

The squid giant axon has provided a unique and powerful model system in which to study the physiology of axoplasmic transport. Indeed, the original observation of microtubule-based transport was made in squid axons (Brady et al., 1982), while biochemical identification of the first microtubule-based motor, kinesin, was achieved using squid optic lobe (Vale et al., 1985). Subsequently, squid axoplasmic organelles were shown to move on actin filaments, implicating myosins as additional transport motors (Kuznetsov et al., 1992; Bearer et al., 1993; Langford et al., 1994). Although the myosin superfamily is relatively well characterized, those myosins that associate with organelles have not yet been well defined.

In squid only two myosins, myosin V and siphon muscle myosin II, have been previously identified by sequence analysis (Medeiros et al., 1998; Matulef et al., 1998; Molyneaux et al., 2000). One of these, myosin V, has been localized to the endoplasmic reticulum (ER), and ER movements are blocked by a peptide antibody raised against the tail domain of this myosin (Tabb et al., 1998; Molyneaux et al. 2000). Thus, myosin V appears to play a role in ER trafficking. In addition, there is evidence that at least one other myosin is involved with organelles. Our earlier studies demonstrated that an anti-myosin antibody detected another protein, larger that myosin V, which co-purified with organelles. (Bearer et al., 1993). This myosin antibody also labeled organelles by immunocytochemistry, suggesting that this second myosin, in addition to myosin V, is associated with axoplasmic organelles.
This second myosin has proven difficult to characterize for a number of reasons. First, the organelle fraction contains insufficient amounts of protein to obtain peptide sequences that could be useful in its identification. The relatively small amounts of protein in axons limit biochemical characterization. In fact, conventional squid kinesin, the founding member of this diverse family of motors, could not be obtained in quantity from axoplasm but had to be biochemically purified and characterized from optic lobe (Vale et al., 1985). Second, the anti-scallop muscle myosin II antibody was not a reliable tool for the identification of this ~220 kDa myosin. Although it recognized one prominent band of ~220 kDa in a preparation of isolated organelles, in the axon it recognized at least five other bands by Western blot.

Here, we apply a strategy similar to that used to isolate and characterize squid kinesin (Vale et al., 1985). First extract myosins from optic lobe, which provides sufficient amounts of neural tissue, and then develop probes for these myosins which can be applied to the axon. A high molecular weight protein was obtained from optic lobe using a modification of an established myosin purification protocol. This protein was identified as a myosin through sequence analysis, and probes were generated to determine its presence in axoplasm and study its distribution in the neuron.
MATERIALS AND METHODS

Materials

Squid (*Loligo pealei*) were obtained live by the Marine Resources Center, Marine Biological Laboratory, Woods Hole, MA. Oligonucleotides were synthesized by Integrated DNA Technologies, Coralville, IA. DNA sequencing of plasmid mini-preps was performed by Davis Sequencing, Davis, CA. For all experiments squid optic lobes and axons were dissected and stored in liquid nitrogen until use. Anti-neurofilament antibody was a generous gift from Philip Grant, National Institutes of Health, Bethesda, MD (Grant *et al.*, 1995).

Purification of Squid Non-muscle Myosin II

Squid non-muscle myosin II was purified using a protocol modified from See and Metuzals (1976). Each step of the purification was carried out on ice or at 4°C. Squid optic lobes (100 grams) were thawed in 4 volumes of ice cold high salt buffer (0.6 M KCl, 3 mM beta-mercaptoethanol, 5 mM MgCl$_2$, 20 mM imidazole, pH 7.0) containing a protease inhibitor cocktail (10 mM benzamidine, 10 mM leupeptin, 10 mM pepstatin A, 10 mM aprotinin and 10 mM phenanthroline), and the mixture homogenized by hand using a glass dounce homogenizer. The homogenate was stirred for 30 min followed by centrifugation at 30,000 X g for 15 min. The resulting supernatant was clarified by high-speed centrifugation at 100,000 X g for 90 min. The high-speed supernatant was diluted to a 0.1 M KCl final concentration by adding 5 volumes of ice cold 2 mM MgCl$_2$ and the pH of the solution adjusted to 6.4 with 1 M potassium acetate buffer (pH 4.8). The sample was stirred for 15 min to precipitate actomyosin, and the precipitate pelleted by centrifugation at 30,000 X g for 15 min. The pellet was resuspended in 5 ml of S-500 buffer (25 mM HEPES, 600 mM NaCl, 5 mM MgCl$_2$, 2 mM EGTA, 2 mM DTT, pH 8.0) in the presence of 10 mM ATP and dounced with a 10 ml homogenizer. The suspension was clarified at 100,000 X g for 1 h and the resulting supernatant (S4) placed over a 1.5 X 100 cm, Sephacryl-500 gel filtration column (Amersham Pharmacia Biotech, Piscataway, NJ). Fractions (3 ml) were collected at a flow rate of 0.2 ml/min. Samples of each purification step were analyzed by Coomassie-stained SDS-PAGE.
Peak fractions containing a ~220 kDa myosin were pooled. Peptide sequences were obtained from the purified neural myosin by excising bands from SDS-PAGE gels, followed by limited proteolysis and Edman degradation as previously described (Medeiros et al., 1998).

**Cloning and Sequencing of Squid Non-muscle Myosin II**

The full-length open reading frame of the squid non-muscle myosin II cDNA was determined by seven overlapping clones obtained by PCR. PCR primers were designed to amplify squid non-muscle myosin II transcripts. These primers were based on conserved sequences in the myosin ATP and actin-binding sites and on peptide sequences obtained from purified squid non-muscle myosin II. Two primers 5’-CAYTTYGTNCNGNTGYATN-3’ (sense) and 5’-TCGATCTGGGTGATTTGAGTTG-3’ (antisense) (Y = T/C, N = A/C/T/G) yielded a single band of ~1.2 kb as determined by ethidium bromide stained agarose gel electrophoresis using a 1 kb ladder as size standard (Gibco BRL, Grand Island, NY). The PCR product was cloned using an Invitrogen TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) and 10 of the resulting clones screened for a 1.2 kb insert by Eco-RI digestion. Two positive clones were sequenced in both the forward and reverse directions using M13R and M13F primers that bind sequences internal to the vector and that flank the insertion site. Full-length sequencing of each of these inserts revealed two identical 1,139 bp sequences. By fasta searches using gcg software the insert sequences were determined to share sequence identity with other non-muscle myosin IIs in the Genebank database. This initial cDNA was extended by a series of PCR reactions using a combination of gene specific primers, primers based on non-muscle myosin II consensus sequences and standard techniques in the rapid amplification of cDNA ends (RACE) (Gibco BRL, Grand Island, NY). These primers are listed in Figure 2 and their position relative to the full-length non-muscle myosin II cDNA sequence shown in Figure 3.

**Antibody Production**
A 927 bp DNA fragment encoding a 309 aa sequence of the squid non-muscle myosin II heavy chain (amino acids 737-1046) was ligated in frame into a Qiagen expression vector to create a His tag construct (Qiagen Inc., Chatsworth CA). The recombinant non-muscle myosin II (rNMMII\textsuperscript{737-1046}) was purified using a standard protocol (Qiagen, Chatsworth, CA). The rNMMII\textsuperscript{737-1046} was further purified by electrophoresis using a 12% SDS-PAGE curtain gel and the resulting protein band excised from the gel and used as the immunogen for antibody production in rabbits. Antisera were screened and affinity purified on rNMMII\textsuperscript{737-1046} affinity columns.

Antibodies to myosin V were also generated. A single peptide sequence, which was obtained from purified squid myosin V (DeGiorgis, Reese and Bearer, unpublished results) that maps to the myosin V head domain (\textsuperscript{195}KVLASNPIMESIGNAK\textsuperscript{211}) was synthesized, coupled to ovalbumin and used as an antigen to produce a polyclonal antibody in rabbit (Covance, Oakland, CA). Antisera were affinity purified against the peptide sequence by column chromatography. The affinity purified anti-myosin V antibody (\(\alpha\)MV) recognized 0.5 \(\mu\)g of purified myosin V at a 1:2000 dilution.

**Preparation of Squid Optic Lobe Homogenates and Axoplasm for Western Blot Analysis**

An optic lobe homogenate was prepared by homogenizing 5 grams of thawed squid optic lobes in 6 volumes of 1/2X buffer (Schnapp \textit{et al.}, 1992) containing 10 mM of each of the following protease inhibitors: benzamidine, leupeptin, pepstatin A, aprotinin and phenanthroline. The homogenate was aliquoted into 1.5 ml eppendorf tubes and centrifuged at 14,000 RPM for 10 min at 4\textdegree C. The resulting supernatant was drawn off and saved as the homogenate. Squid axoplasmic samples were prepared by extruding axoplasm from 10 thawed axons (approximately 50 \(\mu\)l) into 5 volumes of 1/2X buffer containing protease inhibitors. Gel sample buffer (6X) was added to each sample to a 1X final concentration (0.0625 M Tris (pH 6.8), 10% glycerol, 1% SDS, 1% beta-mercaptoethanol, 0.05% bromophenol blue) and the samples boiled for 5 min prior to gel electrophoresis.
**Coomassie Gels and Western Blot Analysis**

For Western blots, proteins were transferred from 8.5% acrylamide gels onto nitrocellulose membranes. The membranes were blocked in 5% powdered milk in 1X Tris buffered saline (TBS) pH 7.4 for 1 h at room temperature. Primary antibodies were diluted to 1:500 in wash buffer (3% powdered milk, in TBS containing 0.2% Tween-20, pH 7.4), and the nitrocellulose blots incubated for 90 min at room temperature in primary antibody solution. Blots were washed 3 X 10 min in wash buffer and then incubated in wash buffer containing 1:5000 alkaline phosphatase conjugated anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN). Blots were washed 3 X 10 min in TBS and developed with NBT/BCIP solution (Kirkgaard and Perry Laboratories, Gaithersburg, MD).

**RT-PCR Assay for Squid Non-muscle Myosin II Transcripts in Squid Neural Tissues**

The expression of non-muscle myosin II transcripts in squid neural tissues was assayed using tissue-specific cDNAs and gene specific primers. Total RNA was extracted from squid optic lobe and stellate ganglia using the Trizol Reagent method (Gibco BRL, Grand Island, NY). RNA (2 µg) was treated with 2 units of amplification grade DNAse I (15 min at 25°C) in 10 µl of 1X reaction buffer (20 mM Tris-HCl (pH 8.4) 50 mM KCl, 2 mM MgCl₂) to remove potential genomic DNA contamination (Gibco BRL, Grand Island, NY). The DNAse I was heat inactivated at 65°C for 15 min in the presence of 2 mM EDTA. RNA (0.5 µg) was reverse transcribed with random hexamers and Superscript II reverse transcriptase at 42°C for 50 min, and then treated with 1 µl RNAse H mix at 37°C for 30 min (Gibco BRL, Grand Island, NY). The resultant cDNA was used as template for PCR reactions along with two squid, non-muscle myosin II, gene specific primers. These primers correspond to a unique sequence in the squid non-muscle myosin II tail domain (nt 4878-5239; 361 bp product) 5’-CTTGAACCAATTGTCTGAGCAACTG-3’ (sense) and 5’CCAACAGGTCTTTCTAATTCCG-3’ (antisense). As a positive control, PCR reactions were carried out with gene specific primers for squid kinesin (nt 397-801; 404 bp product) 5’-
ATATCGTCCTCAAAACAACGCC - 3’ (sense), 5’- CTCCAAGTTTTCGTCCATTCC - 3’ (antisense) and actin (608 bp product) 5’-GGAGAAGATCTGGCATCACACC- 3’ (sense), 5’- GAAGTTCCCTTCGAAACGAAAGG- 3’ (antisense). Parallel PCR reactions were carried out for each primer set using cDNA in which reverse transcriptase was omitted from the first strand reaction. PCR reactions were performed in a Perkin-Elmer thermocycler at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min using 30 amplification cycles. PCR products (20 µl) were separated on 1% agarose gels containing ethidium bromide and photographed at f-22 for 1 sec. Squid non-muscle myosin II PCR products from both optic lobe and stellate ganglia were cloned using an Invitrogen TOPO TA cloning kit (Invitrogen, Carlsbad, CA), mini-preped with Qiagen plasmid mini-prep kit (Qiagen Inc., Chatsworth, CA) and sequenced to verify their identity.

**Axoplasmic Organelle Isolation**

Squid axoplasm was extruded from 10 axons (~50 µl) into 75 µl of 1/2X buffer containing protease inhibitors (as above) and brought to 0.6 M KI with a 3 M KI stock. The solution was triturated 50 X with a wide bore pipette and place on ice for 10 min to dissociate the cytoskeleton. The resulting homogenate was diluted 1:1 in 1/2X buffer, layered over a sucrose step gradient (100 µl of 45% sucrose, 200 µl of 15% sucrose and 100 µl of 12% sucrose in 1/2X buffer) and centrifuged at 35,000 RPM for 1.5 h at 4°C in a Beckman SW 55.1 rotor (Beckman, Fullerton, CA). The supernatant and each sucrose layer were removed with a syringe by puncturing the side of the centrifuge tube. Fractions were analyzed by SDS-PAGE and Western blots as well as by immuno-electron microscopy. Quantitative analysis of Western blots was carried out using a Bio-Rad Gel Doc and Quantity One software (Bio-Rad Laboratories, Hercules, CA). Protein concentration of axoplasmic sucrose fractions was determined using a standard Bradford protein assay.

**Immunogold Labeling of Axoplasmic Organelles**
Glow-discharged carbon-Formvar-coated grids were placed on droplets of KI-washed isolated axoplasmic organelles for 1 min to allow organelles to adhere to the coated surface. The grids were blocked in 10 mg/ml bovine serum albumin (Sigma, St. Louis, MO) and 2% fish gel (Tedd Pella, Redding, CA) in 1/2X buffer for 30 min followed by incubation in 1:200 dilution of anti-squid non-muscle myosin II antibody (αMII) in blocking solution for 90 min. The grids were washed in blocking buffer (3 X 10 min) and incubated in a 1:10 dilution of protein-A gold (Amersham Pharmacia Biotech, Piscataway, NJ) in blocking buffer for 1 h. Grids were washed 3 X 5 min in TBS and negative-stained in 1% uranyl acetate in water. Grids were processed in parallel but in the absence of primary antibody to determine non-specific decoration by protein-A gold.

Six independent experiments were carried out using a variety of blocking agents. The combination of bovine serum albumin and fish gel yielded the least amount of background labeling and resulting grids were used for statistical analysis. Areas of the grids were selected based on optimal negative stain and organelle density for visualization. Fields of organelles were photographed at 20,000 X in a JEOL CX 200 and gold particles counted on randomly selected micrographs. Gold particles were considered to be associated with an organelle if the particle was on the surface of the organelle or found within a 10 nm distance (diameter of one gold particle) of the organelle. The number of gold particles associated with each organelle and those in the background were counted and the area occupied by organelles and background determined.
RESULTS

Purification and Identification of Squid Non-muscle Myosin II

A protocol designed to purify myosin was used to extract a high molecular weight myosin from squid optic lobe (Figure 1) (See and Metuzals, 1976; Medeiros et al., 1998). By differential centrifugation a high-speed supernatant (Figure 1, S4) was obtained that was enriched for a protein (p220) migrating at ~220 kDa, a molecular weight similar to that of other myosin heavy chains. This protein was further purified from the high-speed supernatant by gel filtration chromatography (Figure 1, GF68). From 100 grams of squid optic lobe, approximately 0.4 mg of p220 was obtained. The peak fraction contained p220 at a concentration of approximately 100 µg/ml.

Seventeen peptide sequences were obtained from purified p220 by Edman degradation sequencing (Table 1). These peptides sequences range in length from 7 to 25 amino acids and constitute a total of 210 residues. Eleven of the seventeen peptide sequences match other myosin sequences in our myosin database by fasta search using ggc software (Medeiros et al., 1998). The other six peptides are not identifiable by Blast of the NCBI databank or fasta searches of our myosin directory.

Primers based on p220 peptide sequences and on conserved myosin consensus sequences were used to obtain a 1,139 bp myosin fragment by RT-PCR of squid optic lobe total RNA (Figure 2, clone 1). This initial clone was extended in both the 5’ and 3’ directions by a series of PCR reactions and standard techniques in the rapid amplification of cDNA ends (RACE). Seven overlapping clones encode a 5,892 bp open reading frame and 5’ and 3’ untranslated regions (Figure 2A). Comparison of the full-length deduced amino acid sequence to sequences in the NCBI databank identifies this protein as a squid non-muscle myosin II heavy chain. This sequence most closely matches the sequences of the non-muscle myosin II isoforms of Drosophila melanogaster (58.9%) (zipper gene, Accession No. A36014) and Caenorhabditis elegans (57.1%) (Accession No. T16416).

All seventeen peptide sequences originally obtained from p220 were found to match squid non-muscle myosin II (Figure 3). Four of the peptide sequences (No. 3, 5, 6, 9) map to the myosin
motor domain, two peptides (No. 2, 8) map to the myosin neck domain and the remaining eleven sequences map along the tail domain from amino acids 1175-1834. Those sequences previously unidentifiable by Blast and Fasta searches each map to less conserved regions of the non-muscle myosin II tail domain. These results verify that we have cloned the cDNA that encodes p220 and identify p220 as a squid non-muscle myosin II (sqNMMII).

**Specificity of Non-muscle Myosin II Antibody**

A squid non-muscle myosin II antibody (αMII) was generated against a unique recombinant 309 aa sequence (737-1046aa) of the sqNMMII heavy chain over-expressed in E. coli (rNMMII\textsuperscript{737-1046}) (Figure 3). The rNMMII\textsuperscript{737-1046} includes part of the myosin head domain, the myosin neck domain which encompasses the IQ motifs, and part of the proximal tail in the region predicted to form a coiled-coil. The rNMMII\textsuperscript{737-1046} does not contain the highly conserved ATP or actin-binding sites or other highly conserved regions of the myosin head domain or tail.

The region of sqNMMII we chose as an immunogen is significantly different from squid myosin V, the only other myosin known to be present in squid neural tissue (Figure 4; Table 2). Comparison of the rNMMII\textsuperscript{737-1046} sequence to squid myosin V (Accession No. AAF12809) reveals a sequence identity of only 23.3% (Table 2, upper panel). In contrast a comparison to another non-muscle myosin II from *Drosophila* reveals a 61.3% sequence identity (Table 2, upper panel). Sequences of rNMMII\textsuperscript{737-1046} and squid myosin V were compared to determine whether there are local regions of homology (Figure 4). Only a short sequence of 22 amino acids (758-779 aa) revealed any degree of similarity (63.3%) (Figure 4, underlined).

As a further test that the rNMMII\textsuperscript{737-1046} encoded a domain unique to class II myosins, we also compared this domain to other myosin heavy chains. We could not do an extensive sequence comparison in squid since in this species only two myosin heavy chain genes have been previously sequenced. We therefore used the *Drosophila* database to compare the homologous rNMMII\textsuperscript{737-1046} domain of the *Drosophila* non-muscle myosin II gene (zipper) to other myosins in the *Drosophila* genome. We reasoned that the degree of similarity between this domain of the zipper gene and
other myosin sequences in the Drosophila genome would be similar to the degree of sequence homology between different myosin sequences within the genome of the squid. For instance, the sequence identity between rNMMII\textsuperscript{737-1046} and squid myosin V is 23.3% and to squid muscle myosin is 37.7%. This same domain in the Drosophila zipper gene is 23.0% identical to Drosophila myosin V and 34.8% identical to Drosophila muscle myosin II (MHCII) (Table 2). The Drosophila genome makes the sequence of the complete set of Drosophila myosins in the genome available (Yamashita et al., 2000). Comparison of the domain homologous to rNMMII\textsuperscript{737-1046} from Drosophila non-muscle myosin II (zipper gene) with other myosins in the Drosophila genome shows that it has less than 27% sequence identity to any other unconventional myosin isoforms. Only the conventional myosins share a high degree (34.8-37.7%) of identity with the non-muscle myosin II antibody domain (Table 2: lower panel). Thus, the rNMMII\textsuperscript{737-1046} domain provides a reasonably unique antigen for antibody production against non-muscle myosin II isoforms.

The resultant anti-non-muscle myosin II antibody (αMII) recognized purified sqNMMII (Figure 5A) as well as a single band of the same molecular weight in optic lobe homogenates (Figure 5B). The fact that αMII recognizes only a single band in optic lobe, the original source of purified sqNMMII, demonstrates that the antibody is specific for sqNMMII, since the optic lobe contains at least one other myosin isoform (myosin V; Tabb et al., 1998). The optic lobe would be expected to contain many, as yet unidentified, myosin isoforms including myosins of glial cells as well as those of endothelial and smooth muscle cells from blood vessels (Murakami and Elzinga, 1992).

The specificity of αMII was further tested by probing optic lobe homogenates in parallel with either αMII or αMV (anti-squid myosin V) (Figure 5B). We also generated the αMV antibody against a unique peptide sequence that maps to the myosin V head domain (see Materials and Methods). While αMII detects a band of 220 kDa in optic lobe homogenates, the αMV recognizes a lower band at ~196 kDa. A lane probed with both antibodies shows two discrete
bands (Figure 5B), demonstrating that these are different myosins and that each is recognized specifically by its respective antibody.

**Expression of Non-muscle Myosin II in the Stellate Ganglion**

Proteins present in the giant axon are synthesized in the neuronal cell bodies of the stellate ganglion. PCR has been successfully applied to probe for myosin expression across tissue types in other organisms (Itoh and Adelstein, 1995). Thus we used specific non-muscle myosin II primers in RT-PCR reactions to assay for sqNMMII transcripts in the stellate ganglion. SqNMMII transcripts were detected in both the squid optic lobe and the stellate ganglion (Figure 6). Only samples prepared with reverse transcriptase produced products demonstrating that product was generated from message and not genomic template. Each 361 bp product was cloned and sequenced to verify that it was sqNMMII. RT-PCR experiments using specific primers for kinesin and actin demonstrate that transcripts for each of these proteins are expressed in optic lobe and the stellate ganglion.

**Squid Non-muscle Myosin II Co-purifies with KI-stripped Axoplasmic Organelles**

To determine whether sqNMMII is present in the axoplasm of the squid giant axon, Western blots of extruded axoplasm were probed with αMII (Figure 7). Squid optic lobe homogenate was electrophoresed and blotted in parallel with axoplasm. In both samples, a ~220 kDa band was detected for sqNMMII (Figure 7A).

To determine whether sqNMMII associates with axoplasmic organelles, we fractionated the axoplasm according to a protocol that involves incubation of axoplasm for 10 min in 0.6 M potassium iodide (KI) (Schroer et al., 1988). This KI treatment solubilizes the cytoskeleton enabling for the subsequent separation of organelles from other axoplasmic proteins by sucrose density gradient fractionation. These organelles have been shown to translocate towards the plus ends of microtubules (Schnapp et al., 1992) as well as along filamentous actin (Bearer et al.,
1996a). The KI step is believed to strip cytoplasmic dynein and other loosely associated proteins from the organelles but does not remove kinesin (Schnapp et al., 1992). The αMII recognized a single band of ~220 kDa in the sucrose fraction containing KI-stripped organelles (Figure 7B). This band is the same apparent molecular weight as the band recognized in both the axoplasmic and optic lobe samples. Thus, it appears that sqNMMII is present in all three samples.

Quantitative analysis demonstrates that 43.2% of the total sqNMMII co-purifies with organelles (Fig. 8; Table 3). Equal volumes (10 ul) from each step in the gradient were separated by gel electrophoresis and probed in parallel for either sqNMMII or neurofilament protein. The Western blot band intensities for each protein were measured by densitometry. The percentage of the total was calculated for each sucrose fraction taking into account the fraction volume. SqNMMII was only detected in the supernatant and organelle fractions. The supernatant contains the remainder (56.8%) of the total sqNMMII. Protein concentration measurements show that the supernatant and the 12% fraction contain the majority of the total protein (92.8%) with only 1.6% in the organelle fraction. Thus, sqNMMII is enriched 15.5-fold in the organelle fraction as compared to the supernatant fraction. In comparison, the intensity of the neurofilament band follows a pattern similar to that of the protein concentration, highest in the supernatant and decreasing in concentration down the gradient.

**Squid Non-muscle Myosin II Associates with Isolated Axoplasmic Organelles**

Co-sedimentation with organelles suggests that this myosin is directly attached to organelles. To test whether sqNMMII was indeed associated with the axoplasmic organelles, organelles were stained with αMII/immunogold and examined by electron microscopy (Figure 9A, C). In ten fields, 11% of organelles were labeled with a single gold particle, while 10% were labeled with two or more particles (Figure 9B). Thus, 21% of all organelles were decorated by immunogold.
Less than 1% of the organelles were labeled in grids stained with protein-A gold in the absence of primary antibody.

We quantified the number of particles in the area occupied by organelles as compared to the number of particles occurring in the unoccupied (background) regions of the grids (Table 4). The frequency of particles associated with organelles was 6.01 particles/µm². In contrast, the background area had only 0.24 particles/µm². Thus, the area occupied by organelles has a 25-fold increase in gold particles as compared to background. In the absence of primary antibody, no increase in association of gold particles with organelles was observed. With protein-A gold alone, the frequency of gold particle on organelles (0.32 particles/µm²) was similar to background levels (0.27 particles/µm²) (Table 4). Labeled organelles varied in size and shape, from the smallest vesicles in the preparation, measuring ~50 nm, to the large mitochondria-like organelles, measuring ~500 nm (Fig. 9C). The surface contours of the labeled organelles also varied. Thus, sqNMMII appears to associate with a wide variety of organelles as determined by these morphological criteria.
DISCUSSION

The giant axon of the squid is a powerful model in which to study the physiology of axonal transport, but poses significant difficulties for biochemical identification of the motor proteins involved. We initially reported that a myosin-like protein co-purifies with motile organelles from the giant axon (Bearer et al., 1993), but until now have not been able to definitively identify this protein. Small amounts of this myosin could be obtained from axoplasm, but the protein was too large and the yield was not sufficient for peptide sequencing (Bearer et al., 1996b). Identification by mass spectroscopy also was not feasible, since only two squid myosin sequences have been entered into the databank.

In this paper, we characterize this myosin and show that it is tightly associated with axoplasmic organelles. The present approach starts by obtaining proteins from the optic lobe, the largest structure of the squid central nervous system, with the expectation that proteins expressed in the optic lobe would include those also present in the giant axon. The optic lobe yielded a purified squid myosin (p220) that has provided peptide sequences (Table 1). Cloning and sequencing of the cDNA that encodes p220 allowed us to classify it as a non-muscle myosin II (sqNMMII) according to the standard criteria for myosins, which is based on the amino acid sequence of the head domain (Goodson and Spudich, 1993; Cheney and Mooseker, 1993; Berg et al., 2001). The full-length sequence also allowed us to develop specific tools, including an antibody specific to non-muscle myosin II and primers for PCR, to identify this myosin in other cells and tissues.

Is the Optic Lobe Myosin II Present in Axons?

Our data provide reasons to believe that this optic lobe non-muscle myosin II is also present in the axon. First, RT-PCR demonstrates that the mRNA for sqNMMII is expressed in the cell bodies that give rise to the giant axon. This suggests but does not prove that the protein detected in the
axon is sqNMMII. The proteins present in the axon are synthesized in the cell body however, some of these proteins may not enter the axon proper. Some of the proteins expressed in neurons might remain solely in the cell body or may localize to other regions of the cell such as dendritic processes. RT-PCR has been employed in other species to detect tissue specific expression of non-muscle myosin II, and it has been found to be ubiquitously expressed (Itoh and Adelstein, 1995).

Secondly, by Western blot analysis our antibody raised against a specific domain of sqNMMII (αMII) recognizes a single band in optic lobe homogenates as well as a band of the same molecular weight in squid axoplasm. This antibody was generated against a unique 309 amino acid domain that is highly specific for this myosin II and the antibody does not cross react with myosin V. Furthermore, this antibody is unlikely to recognize myosins of other classes, since these isoforms are equally or more divergent than myosin V from non-muscle IIs in the domain used for antibody production (aa 737-1046).

Even though the Drosophila genome, the closest to squid of the completed genomes, contains only a single non-muscle myosin II gene, we cannot rule out the possibility that there are other non-muscle myosin IIs in squid. There could also be minor molecular differences between the myosin II cloned from optic lobe and the protein present in axons. The axon could contain different splice forms since myosin IIs are known to be alternatively spliced (Kelley and Adelstein, 1995), although whether splicing affects function is not clear. It has been difficult to differentiate splice forms by SDS-PAGE or Western blot even with peptide antibodies against spliced-in amino acid sequences. Other approaches will be necessary to determine whether small sequence variations exist in the axoplasmic non-muscle myosin II isoform.

**Guilt by Association?**

Two lines of evidence provide strong support for a physiological association of sqNMMII with
the cytoplasmic surface of axoplasmic organelles. First, 43.2% of the total sqNMMII remains associated with organelles even after stripping with 0.6 M KI. Similarly, the microtubule motor kinesin is not stripped with KI from organelles (Schnapp et al., 1992). Furthermore, by immunogold immunocytochemistry sqNMMII is detected on the cytoplasmic surfaces of intact organelles indicating that this myosin is exposed and thus available to serve as a motor and is not sequestered inside the organelle to be deployed later at some distant site. Such KI-stripped organelles are known to be motile on both actin filaments and microtubules (Bearer et al., 1996a; Schnapp et al., 1992).

This association between sqNMMII and axoplasmic organelles is strong, evidence for a functional role in organelle transport. Association of motors with organelles as demonstrated by various immunological and biochemical techniques has proved to be a reliable predictor of function. Localization of kinesin after sequential extractions in cultured cells provided evidence of its role in vesicle transport (Morris and Hollenbeck, 1995). Immuno-fluorescence detection has served to identify the subcellular location of kinesin isoforms and thereby to differential their cellular function (Signor et al., 1999; Henson et al., 1992). Finally, immunogold labeling of axoplasmic and cellular organelles with antibodies specific for different kinesin isoforms has contributed to our understanding of which of this large superfamily are associated with organelles (Yamazaki et al., 1995; Moreira et al., 1998).

The strongest evidence of a role for sqNMMII in transport given the tools available is the biochemical association demonstrated here. Blocking antibodies and genetic knockout experiments can be used to determine whether a motor is involved in organelle transport (Yang et al., 2001; Sandberg et al., 2000; Doberstein et al., 1993). However, these approaches have significant drawbacks, especially in squid. For example, inhibitory antibodies are difficult to generate and antibody-blocking experiments can be hard to interpret. Although one myosin V antibody has been reported to block organelle movements in diluted axoplasm, another has no effect (Tabb et al.,
1998). In the squid model system, genetic mutations are not yet an option. Now that myosin II has been found associated with organelles, studies in other organisms in which genetic approaches are possible can be initiated.

**Could sqNMMII be membrane associated?**

That the myosin associated with organelles is a member of the myosin II subgroup comes as a surprise, since myosin IIs are generally thought to form multimeric anti-parallel thick filaments that pull actin filaments against each other as in the classic Huxley model of contraction (Huxley and Simmons, 1971). The myosin II subgroup includes striated muscle, smooth muscle and non-muscle isoforms. The muscle myosins each have highly defined roles in muscle contraction and it is well documented that non-muscle myosin IIs are involved in a variety of contractile processes including cell division, cell motility, and chemotaxis (Straussman et al., 2001). However, a number of findings suggest that not all myosin IIs self-associates into thick filaments and some may mediate functions other than contractility.

Evidence suggests that non-muscle myosin II may take on a variety of structural conformations. The non-muscle myosin II from *Drosophila* (zipper gene), has been shown to form short dumb-bells by rotary platinum shadow electron microscopy (Kiehart and Feghali, 1986). This myosin II is required for membrane movements during closure of the amnioserosa in *Drosophila* embryos. It could associate with membranes via the middle bar of the dumb-bell. In our early studies, electron microscopy of an axoplasmic myosin revealed “flowerettes,” or aggregates of myosins with all globular (head) domains at one end, held together by associations between the sinuous tails (Bearer et al., 1996a). This conformation of myosin multimers could easily support tail-mediated organelle association. Some of the sqNMMII described here could be associated with organelles via an interesting link with other myosin IIs in a thick filament.
conformation. However, evidence for such thick filaments was not found in our electron-microscopic examination of organelles by negative stain. Biochemical studies of non-muscle myosin IIs have shown that thick filament formation is regulated by phosphorylation of the tail domain. For example, the phosphorylation of residues in the non-muscle myosin IIB rod inhibits thick filament formation (Murakami et al., 1995). Phosphorylation mediates membrane association (Murakami et al., 1995). Thus, there may be different structural and functional forms of these myosin II motors, which could be generated by alternative splicing or post-translational modifications.

**Some Non-muscle Myosin IIs are Membrane-associated Motors.**

Emerging evidence links non-muscle myosin IIs with membrane dynamics in a wide range of species and tissue types. Myosin II knock-out mutations in Dictyostelium demonstrate that myosin II is required for agonist-induced rounding (Springer et al., 1994) and surface membrane tension (Jay and Elson, 1995), as well as cytokinesis in suspension (Springer et al., 1994). Actin-based vesicle movement in extracts of clam oocytes is blocked by myosin II-specific antibodies (Sandberg et al., 2000). Immuno-localization of myosin IIs in cultured neurons shows that myosin IIB is found at the leading edge of growth cones as well as in the organelle-rich central region at the end of extending axonal microtubule bundles (Cheng et al., 1992). In the present study, 43.2% of the non-muscle myosin II in axoplasm is associated with KI-washed axoplasmic organelles. Thus, organelle-bound myosin represents a major component of the total non-muscle myosin II in these cells.

**Non-muscle Myosin IIs May Play Other Roles in Neurons.**
In mature neurons, the role of non-muscle myosin II is not well understood. Myosin II in the axon could be mediating vesicle transport on actin filaments. Alternatively, this organelle-associated myosin may be inactive while in transit to the synapse. Myosin II tails tagged by GFP have been shown to localize to the cleavage furrow during cytokinesis. Thus not all myosin reach their final intercellular destinations through their head domain motor activities (Zang and Spudich, 1998). Myosin IIs appear to perform necessary functions in the brain of vertebrates (Wylie and Chantler, 2001; Wylie et al., 1998; Chantler, 1997) where myosin IIB is required for normal brain development in mammals (Tullio et al., 2001). Myosin II could also be involved in maintaining axonal macro-structure through acto-myosin contractions. Axons appear to be under tension, and it has been proposed that this tension is mediated by acto-myosin interactions (Baas and Ahmad, 2001).

ACKNOWLEDGEMENTS

We acknowledge Ben Greenfield for the original sqNMMII cDNA clone, Zhi Li for generating the αMII antibody, and Timna Onigman and Heather Davidson for characterizing the antibody on bacterially expressed protein. We thank Howard Jaffe, for obtaining peptide sequences, and Jennifer Petersen and John Chludzinski for help in the laboratory. We are grateful for the work of Brown undergraduates on this project, including Kendrick Jones, Eric Schneider and Paul George. We thank Louie Kerr, at the Central Microscopy Facility, Marine Biological Laboratory for advice and technical assistance and Jorge Moreira for advice on immunocytochemistry. We would also like to thank Ed Enos and the staff of the Marine Resources Center, Marine Biological Laboratory for the collection and maintenance of squid. Supported by NINDS (TSR) and National Institutes of Health, GM-47368 (ELB), and NSF.
REFERENCES


FIGURE LEGENDS:

Figure 1. Purification of a squid non-muscle myosin II. Coomassie-stained 8.5% SDS-PAGE of optic lobe homogenate (H), supernatants (S) and corresponding pellets (P) of squid optic lobe samples obtained by a series of four centrifugation steps (S1-P4). The final high-speed supernatant, S4 fraction, (indicated by asterisks) is enriched in a high molecular weight protein (p220). The ~220 kDa protein (arrow) was further separated from other proteins by gel filtration of the S4 fraction in the presence of ATP (GF68). Molecular weight markers indicated by dashes on left: 200, 116, 98, 68, 31 kDa.

Table 1. Peptide sequences of purified p220. The p220 myosin was excised from SDS gels and sequenced by Edman degradation (amino acid sequence). “Name” is a list of numbers referring to the order in which sequences were obtained. "Position" refers to map position of each peptide sequence along the predicted amino acid sequence of the non-muscle myosin II (Figure 3). The degree to which each peptide sequence matches the predicted amino acid sequence of non-muscle myosin II is provided (% Identity).

Figure 2. Cloning of squid non-muscle myosin II. (A) Schematic representation of overlapping cDNA clones that encode the full-length squid non-muscle myosin II cDNA nucleotide sequence (Accession No. 406790), and (B) table of sqNMMII PCR primers and their corresponding clones. Clones are numbered in the order that they were acquired (clones1-7). The position along the full-length non-muscle myosin II cDNA of each clone and primer is given as well as their lengths in base-pairs.

Figure 3. Position of sqNMMII peptide sequences and the αMII antibody domain along the full-length sqNMMII amino acid sequence. Peptide sequences are numbered (1-17) and refer to the p220 peptide sequences listed in Table 1. A polyclonal antibody was generated against a 309
amino acid sequence that maps to amino acids 737-1046 of squid non-muscle myosin II (rNMMII^{737-1046}).

**Figure 4.** Amino acid sequence comparison of the rNMMII^{737-1046} antigen sequence to the corresponding domain of the squid myosin V heavy chain sequence. Protein sequences were aligned with the gap command, using Wisconsin Sequence Analysis Package (Genetic Computer Group, Madison, WI). Identical residues are shaded in black and similar residues in gray (BOXSHADE: www.ch.embnet.org).

**Table 2.** Comparison of rNMMII^{737-1046} amino acid sequence and the homologous domain of the *Drosophila* non-muscle myosin II zipper gene with other myosins in the same species. The primary structure of the sqNMMII used to generate the αMII antibody is compared to squid myosin V, squid muscle myosin II and *Drosophila* non-muscle myosin II (zipper) (upper panel). The homologous sequence of rNMMII^{737-1046} in the *Drosophila* zipper gene is compared to other myosins in the *Drosophila* genome. The name, class, length and accession number of each myosin is listed as well as the percent identity.

**Figure 5.** Anti-squid non-muscle myosin II antibody is specific for sqNMMII. Commassie-stained 8.5% SDS-PAGE gel of purified sqNMMII (A, sqNMMII/Gel, coom) and corresponding Western blot (A, sqNMMII/Blot, αMII) with αMII. Comassie stained 8.5% SDS-PAGE gel of optic lobe homogenate (B, squid optic lobe/Gel, coom) and corresponding Western blots (B, squid optic lobe/Blots) with αMV antibody (B, αMV), αMII antibody (B, αMII) and both antibodies (B, both). Molecular weight markers indicated by dashes on left: 200, 116, 98, 68, 45 kDa.

**Figure 6.** SqNMMII is expressed in the stellate ganglion. Ethidium bromide stained 1% agarose gel of PCR products using cDNA from optic lobe and stellate as template (as labeled). Primers are
designed to amplify sqNMMII, actin and kinesin. PCR reactions were carried out using first strand cDNA generated in the presence (+) and absence (-) of reverse transcriptase.

**Figure 7.** SqNMMII is found in axoplasm and co-purifies with axoplasmic organelles. Coomassie-stained 8.5% SDS-PAGE gel of optic lobe homogenate and axoplasm (A, Gel/coom OL, Axo) and corresponding Western blots with αMII antibody (A, Blots/αMII OL, Axo). Coomassie stained 8.5% SDS-PAGE gel of axoplasmic organelle fraction (B, Gel/coom, organelles) and corresponding Western blot with αMII antibody (A, Blots/αMII, organelles). Molecular weight markers indicated by dashes on left: 200, 116, 98, 68, 45 kDa.

**Figure 8.** SqNMMII preferentially fractionates with axoplasmic organelles. (A) Coomassie-stained 8.5% SDS-PAGE gel of axoplasmic sucrose density fractions and (B) corresponding Western blots with αMII antibody (top panel) and αNeurofilament antibody (bottom panel). Each lane is loaded with 10 ul of each sucrose fraction including supernatant (Sup), 12% sucrose fraction (12%), 15% sucrose fraction (15%), and 45% sucrose fraction (45%). The 15% fraction is highly enriched in organelles. Molecular weight markers indicated by dashes on left: 200, 116, 98, 68, 45, 31, 21 kDa.

**Table 3.** Quantitative analysis of sqNMMII in axoplasmic organelle fractions. Axoplasm was separated over a sucrose density step gradient in the presence of 0.6 M KI. Each fraction is listed (sucrose fraction). Protein concentration of each fraction was determined by Bradford assay (Protein/lane (ug)). The proportion of total protein in each sample was calculated (Protein/lane (%)). The proportion sqNMMII and neurofilament (NF) was determined by densitometry. Of the total 43.2% of sqNMMII is found in the 15% sucrose fractions containing KI-washed axoplasmic organelles.
**Figure 9.** SqNMMII associates with KI-washed axoplasmic organelles. (A) Low magnification field of KI-washed axoplasmic organelles labeled with αMII primary antibody and protein-A colloidal gold (scale bar = 200 nm), and (B) histogram of organelle labeling. Histogram shows the number of gold particles per organelle in the presence and absence of primary antibody (n = 581). Montage showing micrographs of representative organelles labeled with anti-squid non-muscle myosin II antibody and colloidal gold. (C) Organelles of various sizes are labeled with one or more gold particle (scale bar = 50 nm).

**Table 4.** Statistical analysis of immunogold labeled KI-washed axoplasmic organelles. The number of gold particles per unit area of KI-washed organelles stained for non-muscle myosin II is compared to background label of this preparation as well as parallel labeling with colloidal gold secondary only (in parentheses). The area measured, gold particles gold particles counted, and percent of gold particles in each spaced are provided.
<table>
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<tr>
<th>Name</th>
<th>Amino Acid Sequence</th>
<th>Position</th>
<th>% Identity</th>
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<td>59-72</td>
<td>100</td>
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<tr>
<td>sequence #6</td>
<td>K*RTTFHRDDIQK</td>
<td>72-83</td>
<td>100</td>
</tr>
<tr>
<td>sequence #9</td>
<td>K*VGRDHVTK</td>
<td>413-421</td>
<td>100</td>
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<td>sequence #5</td>
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<td>558-568</td>
<td>90</td>
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<td>100</td>
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<td>LEVDNLK</td>
<td>1175-1181</td>
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<td>sequence #7</td>
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<td>sequence #14</td>
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<td>sequence #17</td>
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<td>sequence #12</td>
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<td>1735-1750</td>
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<td>sequence #15</td>
<td>K*IASENLEDDLDDQD</td>
<td>1823-1834</td>
<td>92</td>
</tr>
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Where K* = assumed to be K based on the specificity of the enzyme endoproteinase Lys-C
Where X = uninformative residues; S, H = mismatches

Table 1: Peptide sequences of purified p220
<table>
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<th>Name</th>
<th>Class</th>
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<th>% Similarity</th>
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<td>Nina C</td>
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<td>35.6</td>
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<td>23.0</td>
<td>33.0</td>
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<td>95F (jargene)</td>
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<td>25.3</td>
<td>40.4</td>
<td>1253</td>
<td>Q01989</td>
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<td>ck (crinkled)</td>
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<td>22.8</td>
<td>34.7</td>
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<td>26.3</td>
<td>38.5</td>
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<td>AAF34810</td>
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Table 3: Quantitative analysis of non-muscle myosin II in axoplasmic fractions

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<th>Sucrose fraction</th>
<th>Protein/lane (ug)(^a)</th>
<th>Proportion of total Protein/lane (%)(^b)</th>
<th>Proportion of total antigen/fraction (%)(^c)</th>
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<tr>
<td>Supernatant</td>
<td>26.4</td>
<td>53.0</td>
<td>56.8</td>
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<tr>
<td>12%</td>
<td>21.3</td>
<td>42.8</td>
<td>0.0</td>
</tr>
<tr>
<td>15% (organelles)</td>
<td>1.3</td>
<td>2.6</td>
<td>43.2</td>
</tr>
<tr>
<td>45%</td>
<td>0.8</td>
<td>1.6</td>
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</tr>
<tr>
<td>total</td>
<td>49.8</td>
<td>100</td>
<td>100</td>
</tr>
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</table>

\(^a\) Total protein in each gel lane for Coomassie stain and Western blot analysis (10ul of each sucrose fraction)

\(^b\) Proportion of the total protein in each gel lane Coomassie stain and Western blot analysis

\(^c\) Percent of non-muscle myosin II (sqNMMII) and neurofilament (NF) in sucrose density fractions

\(^*\) Percent of antigen/fraction has been adjusted to account for the total volume of each sucrose fraction
**Table 4:** Quantitative analysis of immunogold labeling of KI-washed axoplasmic organelles

<table>
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<th>αMII &amp; secondary antibody</th>
<th>Secondary antibody alone</th>
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<tr>
<td></td>
<td>Area (µm²)</td>
<td># Particles</td>
</tr>
<tr>
<td>Organelles</td>
<td>19.3</td>
<td>116</td>
</tr>
<tr>
<td>Background</td>
<td>333.5</td>
<td>79</td>
</tr>
<tr>
<td>Total</td>
<td>352.8</td>
<td>195</td>
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</table>

* Area on grid—see Methods  
  ^ Gold particles counted
Primer sequences, product size and position relative to the full-length squid non-muscle myosin II cDNA

<table>
<thead>
<tr>
<th>Clone</th>
<th>Sense primer/position/length (bp)</th>
<th>Antisense primer/position/length (bp)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>5'-CAYTTYGTNCGNTGYATN-3' (2023-2040) 18 bp (2)</td>
<td>5'-TCGATCTGGGTGATTTGAGTTG-3' (3141-3162) 22 bp (1)</td>
</tr>
<tr>
<td>2</td>
<td>5'-GCGGAATCGGCTTTAGCAGA-3' (3097-3116) 20bp (3)</td>
<td>5'-NGCYTGRTTTYCNCCYTGYTT-3' (3564-3584) 21 bp (1)</td>
</tr>
<tr>
<td>3</td>
<td>5'-GATACAACTGCTGCTGTCCAAG-3' (3480-3501) 22 bp (3)</td>
<td>5'-GGCCACGCGTCGACTAGTAC-3' 3' RACE primer (4)</td>
</tr>
<tr>
<td>4</td>
<td>5'-GTGCTGAGAATGAGAAATCCC-3' (4406-4426) 21 bp (3)</td>
<td>5'-GGCCACGCGTCGACTAGTAC-3' 3' RACE primer (4)</td>
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<tr>
<td>5</td>
<td>5'-GGNCARAGYGGNGCNGAAR-3' (1240-1260) 21 bp (2)</td>
<td>5'-GGCCTCAACAGAATACTCTGC-3' (2157-2177) 21 bp (3)</td>
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<td>6</td>
<td>5'-GTNGGNNAGRGAYCAYGTNACN-3' (547-567) 21 bp (2)</td>
<td>5'-CGGAATTTGGAACCAAGATCC-3' (1281-1302) 22 bp (3)</td>
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<tr>
<td>7</td>
<td>5'-GGCCACGCGTCGACTAGTAC-3' 5' RACE primer (4)</td>
<td>5'-AGCTTCCAGAATCGGATTGGC-3' (694-714) 21 bp (3)</td>
</tr>
</tbody>
</table>

Where R=A/G, Y=C/T, N=A/C/G/T

* clone 7 encodes the start site and 7 amino acids in the 5’ untranslated region.

Four different types of primers were used as indicated

(1) Degenerate or guessmer based on peptide sequences obtained from purified optic lobe myosin
(2) Degenerate based on amino acid sequences of myosin from other species
(3) Non-degenerate based on cDNA sequences from sqNMMII clones
(4) RACE primers (Gibco-BRL)

The antisense primer sequences are the actual sequences used and are the reverse/complement of the coding sequence.
Sequence comparison: rNMMII $^{737-1048}$ vs. squid myosin V