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# Myosin-X, a novel myosin with pleckstrin homology domains, associates with regions of dynamic actin

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## SUMMARY

**Myosin-X is the founding member of a novel class of unconventional myosins characterized by a tail domain containing multiple pleckstrin homology domains. We report here the full-length cDNA sequences of human and bovine myosin-X as well as the first characterization of this protein's distribution and biochemical properties. The 235 kDa myosin-X contains a head domain with <45% protein sequence identity to other myosins, three IQ motifs, and a predicted stalk of coiled coil. Like several other unconventional myosins and a plant kinesin, myosin-X contains both a myosin tail homology 4 (MyTH4) domain and a FERM (band 4.1/ezrin/radixin/moesin) domain. The unique tail domain also includes three pleckstrin homology domains, which have been implicated in phosphatidylinositol phospholipid signaling, and three PEST sites, which may allow cleavage of the myosin tail. Most intriguingly, myosin-X in cultured cells is present at the edges of lamellipodia, membrane ruffles, and the tips of filopodial actin bundles. The tail domain structure, biochemical features, and localization of myosin-X suggest that this novel unconventional myosin plays a role in regions of dynamic actin.**

Key words: Unconventional myosin, Myosin-X, *MYO10*, Pleckstrin homology domain

## INTRODUCTION

Myosins are actin-based motor proteins that provide the molecular basis for many cellular movements. In addition to the conventional (class II) myosins required for processes such as muscle contraction, cells also express several classes of unconventional myosins. These proteins have motor domains similar to those of the conventional myosins but have tail domains that are structurally unique and confer class specific functions (Mooseker and Cheney, 1995). The physiological importance of myosins has been underscored by the discovery that mutations of these motors can lead to severe phenotypes such as cardiomyopathy, deafness, seizures, and death (Oliver et al., 1999; Sellers, 2000). Thus, each class of myosins appears to be associated with a conserved set of fundamental functions such as contractile filament formation for class II myosins or organelle transport for class V myosins.

We have identified myosin-X, a new member of the myosin superfamily. A partial sequence from the head domain of myosin-X was initially discovered in a PCR screen to identify novel myosins that might be involved in sensory transduction in bullfrog (Solc et al., 1994) and preliminary phylogenetic analysis of the head domain sequence (Mooseker and Cheney, 1995) indicated that it did not fall into any of the known myosin classes. To begin to investigate the structure and function of this putative motor protein, we have now obtained the full-length sequences of bovine and human myosin-X. These sequences constitute a novel class of myosins whose unique tail domain structure includes multiple pleckstrin homology (PH) domains, first identified as ~100 aa modules similar to the N-terminal and C-terminal domains of pleckstrin (Haslam et al., 1993; Mayer et al., 1993). PH domains exhibit only modest protein sequence similarity but fold into a conserved 3-D structure and are found primarily in proteins involved in signal transduction pathways or cytoskeletal function (Lemmon et al., 1996). The PH domains of myosin-X thus raise the possibility of a mechanism by which this novel motor could act as a mobile link between the actin cytoskeleton and numerous signal transduction pathways.

In addition to actin-based motility, unconventional myosins are now known to have roles in signal transduction (Bahler, 2000; Baker and Titus, 1998; Hasson and Mooseker, 1997; Mermall et al., 1998). For example, *Drosophila* ninaC (a class III myosin) is a component of the transducisome, a multi-protein signaling complex required for phototransduction in the *Drosophila* rhabdomere (Li et al., 1998), while class IX myosins possess GTPase activating protein activity for Rho (Muller et al., 1997; Post et al., 1998). Strikingly, class I myosins were recently found to be involved in actin nucleation by the Arp2/3 complex via interactions with the WASP family of proteins (Evangelista et al., 2000; Lechler et al., 2000). These unconventional myosins thus demonstrate the potential for direct connections between actin-based motors and a variety of signal transduction cascades.

Although myosin-X is the founding member of a novel class of myosins, virtually nothing is known about the protein or its function. In this paper we propose a model for the molecular structure of myosin-X and report that it is widely but not abundantly expressed in vertebrate tissues. We present biochemical evidence that myosin-X may associate with actin and cell membranes. We demonstrate that, intriguingly, myosin-X protein is a component of lamellipodia, membrane ruffles, and filopodia. These regions of dynamic actin are widely recognized as substrates for a variety of modes of cell motility, and their structure and regulation is of great interest. The tail domain structure, biochemistry, and localization of myosin-X thus lead us to propose that myosin-X serves as a link between membrane signaling and the actin cytoskeleton.

## MATERIALS AND METHODS

### Isolation, sequencing and analysis of myosin-X cDNAs

A random-primed bovine aorta cDNA library (Lambda Zap, a gift from Dr Tom Michel) and an oligo-dT primed bovine aorta library were hybridization screened using a bullfrog myosin-X PCR fragment (Solc et al., 1994). Plaques that were positive following a tertiary screen were rescued by in vivo excision, and the entire cDNA was sequenced at least threefold. To obtain the human myosin-X cDNA, a human heart cDNA library (oligo-dT and random-primed) was hybridization screened, resulting in a clone of approx. 920 bp at the 5′ end of the human myosin-X cDNA. PCR primers to the 3′ end of this clone and to the 5′ end of the partial cDNA KIAA0799 were used to amplify the intervening sequence using human kidney Marathon-Ready cDNA (Clontech). The resulting approx. 3.3 kb product was sequenced directly and then assembled into a new contig with the previously obtained 5′ clone and KIAA0799. Sequence analysis was performed using the PC/GENE v6.01 (Intelligenetics) and Genetics Computer Group (GCG; Devereux et al., 1984) packages and web-based tools such as Pfam (Bateman et al., 2000) and PESTFIND (Rechsteiner and Rogers, 1996).

### RNA analysis

The cDNA used to probe human multiple tissue northern and master blots (Clontech) was generated by PCR (Forward primer: 5′-CACGAGGCGAATACGTATAAGATCG – 3′; Reverse primer: 5′-CTAAAACCAGCACTGGAGCCAGCTG – 3′) from an expressed sequence tag (EST) corresponding to 150 nucleotides of coding sequence and 850 nucleotides of 3′ untranslated sequence of human myosin-X (GenBank accession # W16936). The probe was labeled by random priming (Boehringer Mannheim), unincorporated counts were removed (ProbeQuant G-50 Micro Columns, Pharmacia Biotech), and probe was added to the hybridization solution to a final concentration of 2-4×$10^6$ cpm/ml. The blots were hybridized and washed using stringent conditions. Autoradiographs were scanned and adjusted for brightness and contrast using Adobe Photoshop.

In situ hybridizations were carried out using frozen sections of mouse brain and testis. Sense and antisense probes spanning nucleotides 4582-4978 of the bovine sequence were labeled with digoxignenin-UTP by T7 and T3 RNA polymerase reactions from a fragment of mouse myosin-X cDNA cloned into pBSK (Stratagene). The sections were hybridized and washed under stringent conditions and developed using reagents from Boehringer Mannheim (100 mM Tris, pH 9.5, 100 mM NaCl, 0.1% Tween-20, 10% polyvinyl alcohol, 50 mM $MgCl_2$, 5 mM levamisol, 0.45% NBT, 0.35% BCIP).

### Antibodies

For biochemical studies and immunolocalization, antibodies were raised from two different fragments of bovine myosin-X. The antigen used for myosin-X head antibodies was a baculovirus-expressed head/neck/coiled coil fragment including aa 1-952 of bovine myosin-X with the point mutation M747T and an 8 aa FLAG tag at the carboxyl terminus, which was used to immunize a rabbit by Covance Labs (Head Ab 117). The antigen used for myosin-X tail antibodies was a bacterially expressed fusion protein consisting of aa 1639-1798 of bovine myosin-X cloned into pGEX-3X (Pharmacia) with glutathione-S-transferase (GST) attached to the amino terminus of the myosin-X sequence. The fusion protein was solubilized in sarkosyl and purified on glutathione beads (Frangioni and Neel, 1993), then used to generate IgY antibodies in chicken serum by East-Acres Biologicals (Tail Ab 1071). Antibodies were purified on affinity columns (Harlow and Lane, 1988) using the fusion proteins against which they were generated, and the tail antibody was affinity depleted to remove immunoreactivity against GST. Non-muscle myosin-IIa polyclonal antibody (BT-561) was purchased from Biomedical Technologies Inc.

### Immunoblots

To assess the specificity of the myosin-X antibodies, bovine tissues were acquired from a slaughterhouse and either frozen immediately in liquid nitrogen or stored on ice until homogenized. Samples of bovine tissue were homogenized at 1 g wet weight/10 ml in buffer containing 40 mM HEPES, 10 mM EDTA, 2 mM DTT, 1 mM Pefablock, 5 mM ATP, 5 μg/ml pepstatin A, pH 7.2, and immediately boiled 5-10 minutes in SDS sample buffer. Samples were separated on 4-20% Tris-glycine SDS-PAGE gels and transferred to nitrocellulose at 150 V-hr in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.02% SDS). Affinity purified antibodies were used at 1-2 μg/ml with 1:100,000 donkey anti-rabbit (Jackson) or 1:2500 donkey anti-chicken (Jackson) secondary. Immunoblots were developed using SuperSignal West Pico chemiluminescent substrate (Pierce) and films were scanned and adjusted for brightness and contrast using Adobe Photoshop.

### Cell culture

Madin-Darby bovine kidney (MDBK) cells were obtained from the Lineberger Comprehensive Cancer Center, UNC-Chapel Hill. MDBK cells were maintained in MEM medium supplemented with 10% fetal bovine serum, 2% glutamine, and 100 U penicillin-streptomycin-fungizone. Lysates were prepared by scraping cells in solubilization buffer (20 mM MOPS, 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.5 mM ATP, 1 mM Pefablock, 0.2 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, pH 7.4) and homogenizing on ice with 75 Dounce strokes. For immunoblotting, crude lysates were immediately boiled in SDS. For actin cosedimentation assays, homogenates were spun in a table-top ultracentrifuge (Beckman) at approx. 150,000 ***g*** for 15 minutes at 4°C. The supernatant was collected and the pellet was resuspended to the same volume.

### Biochemical experiments

For fractionation, bovine kidney was homogenized in solubilization buffer (see above) and subjected to sequential centrifugation (1000 ***g*** for 5 minutes, 10,000 ***g*** for 15 minutes, 100,000 ***g*** for 15 minutes). For in vivo proteolysis, bovine kidney was homogenized in 40 mM HEPES, 2 mM EDTA, 2 mM DTT, pH 7.7 and incubated with 2 mM $Ca^{2+}$ in the absence or presence of 0.5 units of calpain (Sigma). Proteolysis was halted by the addition of EGTA at different time points for analysis by immunoblotting. For actin cosedimentation assays, f-actin was added to MDBK lysates in the absence or presence of 0.2 units of hexokinase (Sigma) plus glucose and incubated for 15 minutes. Samples were then centrifuged at 150,000 ***g*** in a TLA100 tabletop ultracentrifuge rotor for 15 minutes at 4°C and analyzed by immunoblotting.

### Immunofluorescence

MDBK cells were plated onto acid-washed coverslips in twelve-well

cell culture plates for immunofluorescence. The cells were fixed with 3.7% paraformaldehyde for 20 minutes, permeabilized with 0.5% Triton for 10-20 minutes, and blocked in 5% goat serum for 60-90 minutes. Coverslips were incubated with specific antibodies at 5 μg/ml for 1 hour, rinsed three times for 15 minutes in PBS, incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes) at 1 μg/ml for 45-60 minutes, and rinsed in PBS for three 10 minute rinses, one 15 minute rinse containing rhodamine-phalloidin at 1:500 (Molecular Probes), and a final 15 minute rinse. Coverslips were mounted using Prolong mounting medium (Molecular Probes) and examined using a Zeiss fluorescence microscope equipped with a 1.4 NA ×100 objective. Images were obtained using an Orca II cooled CCD digital camera (Hamamatsu) and Metamorph software (Universal Imaging). Confocal images were acquired using a Leica TCS NT system to obtain 0.5-0.7 μm z-sections, which were combined into extended focus images using Metamorph. All images were prepared for publication using Adobe Photoshop.

## RESULTS

### Discovery of bovine and human myosin-X

Three overlapping clones encoding the ~7.8 kb bovine myosin-X coding sequence, ~220 bp of 5′ untranslated sequence, and ~1390 bp of 3′ untranslated sequence (Fig. 1; GenBank accession #U55042) were obtained from an aorta cDNA library. The region surrounding the start codon at bp 223 (ACA***ATG***G) contains seven of eight nucleotides defined by Kozak (1986) as being important for eukaryotic initiation, and there are stop codons in all reading frames of the 5′ UTR. The deduced coding sequence of bovine myosin-X is 2052 aa with a predicted molecular mass of 235,837 Da. The domain structure of myosin-X is depicted in bar diagram form (Fig. 1B). The 3′ UTR begins with a stop codon at bp 6379, extends to a consensus polyadenylation initiator (bp 7753; AATAAA) and concludes at the poly(A) tail (bp 7769).

To identify human myosin-X, we searched the GenBank database and discovered a clone corresponding to roughly the last half of the bovine coding region (KIAA0799; GenBank accession #AB018342). An approximately 2 kb region within KIAA0799 that does not match the bovine cDNA contains an Alu repetitive sequence and is identical to intronic sequence present in genomic sequence for the human *MYO10* gene (GenBank accession #AC010358), suggesting that the KIAA0799 clone results from an incompletely processed mRNA (Fig. 1D). A clone representing the 5′ end of myosin-X was isolated from a human heart cDNA library and a PCR product from human kidney cDNA that spanned the head and tail fragments was identified (Fig. 1D; GenBank accession #AF247457). Human myosin-X consists of 2058 aa with a predicted molecular mass of 237,552 Da, and shares 93% aa identity with bovine myosin-X (Fig. 2). Human myosin-X is represented by over 100 ESTs in GenBank (UniGene entry Hs.61638) and several other full-length sequences for human myosin-X have recently been submitted to GenBank (accession #AF234532 and AF132021/AF132022).

Preliminary intron/exon analysis for the *MYO10* gene was performed using genomic clones from chromosome 5 (GenBank accession #AC010358, AC023060, and AC010607). Based on these contigs, which encompass the entire coding region, the *MYO10* gene spans >200,000 bp and is organized into at least 41 exons (not shown). The identification of genomic sequence from chromosome 5 coding for the human *MYO10* gene confirms prior localization of myosin-X to the region of 5p15.1-14.3 (Hasson et al., 1996). Although no myosin-X

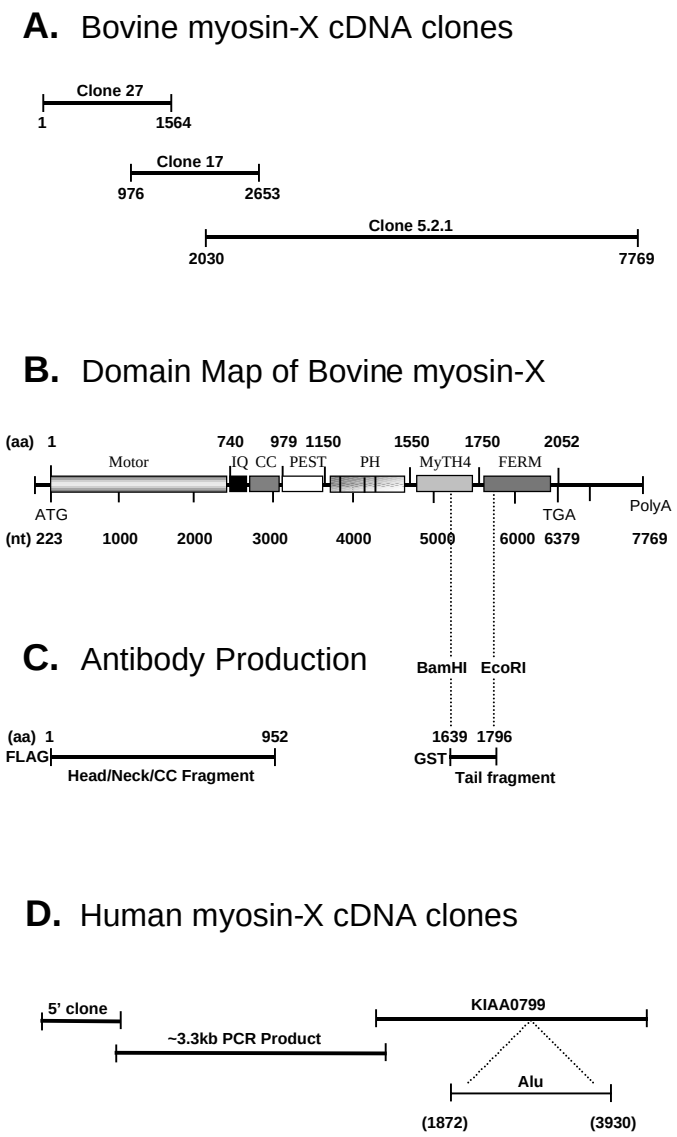


**Fig. 1.** (A) The full length coding sequence for bovine myosin-X was sequenced from three overlapping clones. (B) The deduced sequence indicates that myosin-X contains a conserved motor domain and a neck consisting of three IQ motifs (light chain-binding sites). The tail domain contains a predicted stalk of coiled coil, three PEST regions, three PH domains, a MyTH4 domain, and a FERM domain. (C) A baculovirus-expressed protein fragment consisting of the motor domain, IQ motifs, and coiled coil was used to generate specific antibodies to the myosin-X head. A GST-fusion protein consisting of the region between the MyTH4 and FERM domains was produced in bacteria for generation of specific antibodies to the myosin-X tail. (D) The full length coding sequence for human myosin-X was determined from a partial clone identified in the GenBank database (KIAA0799), a 920 bp clone coding for the 5′ end of the head domain and a ~3.3 kb PCR product. The KIAA0799 clone likely represents an incompletely processed mRNA as it includes a ~2 kb region containing Alu repetitive sequence.

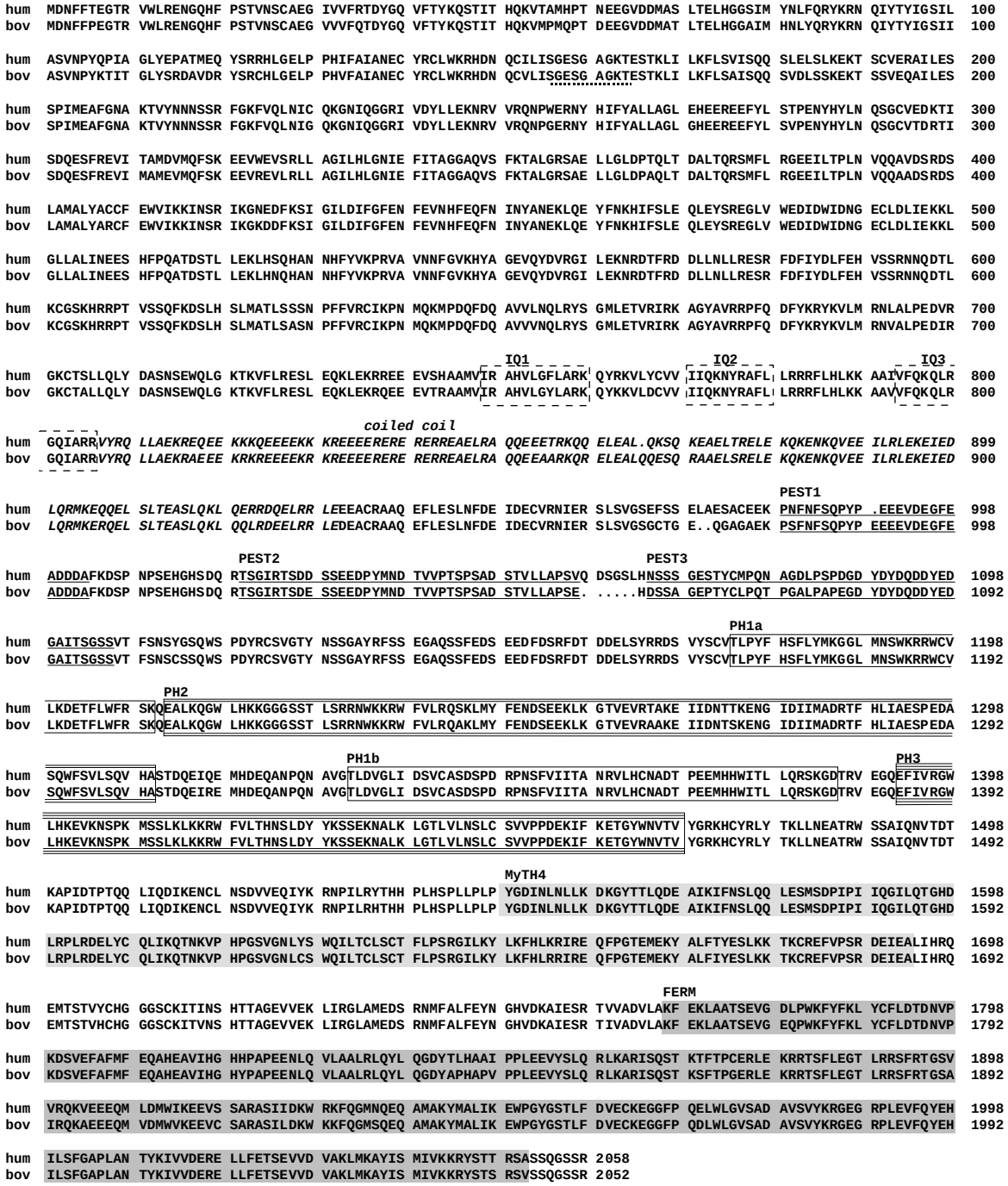


**Fig. 2.** Alignment of human and bovine myosin-X protein sequence. The conserved 'GESGAKT' sequence within the motor domain is indicated by dashed underline. The core portion of the IQ motifs are indicated by dashed boxes, and the region predicted to form coiled coil structure is italicized. The three PEST regions are underlined. PH domains 1, 2 and 3 are indicated by solid boxes with one, two or three lines, respectively. The MyTH4 and FERM domains are shaded in light grey and dark grey, respectively. The myosin-X sequences exhibit 93% sequence identity overall. The sequence data for bovine and human myosin-X are available from GenBank/EMBL/DDBJ under accession # U55042 and AF247457.

mutations have yet been identified, Familial Chondrocalcinosis 2 maps to 5p15.1 (Rojas et al., 1999) and the genetic syndrome known as Cri-du-chat involves variable deletions of the short arm of chromosome 5 (Overhauser et al., 1994).

## Myosin-X is widely expressed among vertebrates

A large number of mouse ESTs (UniGene entry Mm.60590) are present in the GenBank database and a full-length mouse myosin-X sequence (GenBank accession #AJ249706) has recently been reported (Yonezawa et al., 2000). In addition to the initial fragment of frog myosin-X (GenBank accession # U14373), other partial myosin-X sequences are currently known from ESTs of rat (GenBank accession #AI045505; UniGene # Rn.20885) and zebrafish (*Danio rerio*; GenBank

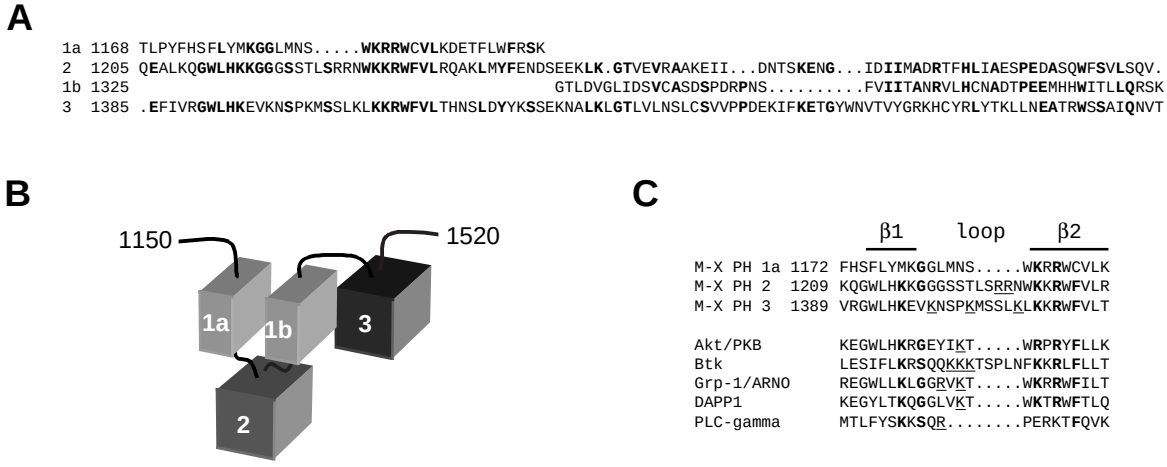


**Fig. 3.** The PH domain complex of myosin-X. (A) Alignment of the bovine myosin-X PH domains indicates that PH1 is relatively well conserved despite the insertion of PH2 into a variable loop region, while PH3 is the least conserved. (B) The organization of PH domains in myosin-X is unique in that the second PH domain is inserted between two halves of the first PH domain. (C) Myosin-X PH domains 2 and 3 contain a number of residues within the β1-loop-β2 region present in other PH domains that have been demonstrated to bind PI3K products. This region has previously been hypothesized to play an important role in mediating binding to $PI(3,4)P_2$ or $PI(3,4,5)P_3$ (Isakoff et al., 1998; Rameh et al., 1997). Conserved residues identified by Isakoff et al., are indicated in bold, and charged residues in the loop are underlined. (GenBank accession #s: Mouse Akt/PKB – X65687, Mouse Btk – L29788, Mouse GRP1 – AF001871, Human ARNO – X99753, Mouse DAPP1 – AF163255, Human PLC gamma – M34667.)

accession #AW078286 and AI601763). Although apparently ubiquitous in vertebrates, class X myosins have not been identified in the complete genomes of *S. cerevesiae*, *C. elegans*, or *Drosophila*. We thus conclude that myosin-X is most likely a vertebrate-specific unconventional myosin.

## Structure of myosin-X

### Head and neck domains

The head domain (bovine aa 1-739) shares 35% sequence identity with the head domain of human skeletal muscle myosin and is most similar (45% identity) to human myosin-VIIa, the gene that is mutated in Usher syndrome type Ib (Chen et al., 1996). Myosin-X is not regulated by phosphorylation at the 'TEDS Rule' site as it contains an acidic residue ($Glu^{383}$) instead of serine or threonine at this site (Bement and Mooseker, 1995). Immediately following the head domain is a neck domain consisting of 3 IQ motifs of approx. 24 aa each. Since IQ motifs provide binding sites for calmodulin or calmodulin-like proteins (Cheney and Mooseker, 1992), we predict that each myosin-X heavy chain is associated with three calmodulin or calmodulin-like light chains.

### Tail domains

#### Coiled coil and a PEST region associated with calpain cleavage

Beyond the IQ motifs is a region predicted to form a coiled coil of 10-20 nm in length (Berger, 1995; Lupas et al., 1991), raising the strong possibility that myosin-X heavy chains form dimers. Between aa 979 and 1115 are three PEST regions, which are enriched in proline, glutamate, serine and threonine and are associated with susceptibility to cleavage by proteases such as the calcium-dependent protease calpain (Rechsteiner and Rogers, 1996). Cleavage of myosin-X at the PEST sites would sever the motor domain from the distal tail domains.

#### Three pleckstrin homology domains

The most unique feature of the myosin-X sequence is the complex of three pleckstrin homology (PH) domains found from aa 1168 to 1491 (Fig. 3A). The PH domains in myosin-X have an unusual organization in that the first PH domain (PH1) is split by the insertion of the second PH domain (PH2) (Fig. 3A,B). This insertion occurs precisely at the site of a highly variable surface loop where other PH domains have also been reported to contain large insertions (Macias et al., 1994; Musacchio et al., 1993). As a result, the two halves of PH1 receive low probability scores individually, but when pieced together PH1 is easily identified by web-based scanning algorithms such as Pfam (Bateman et al., 2000).

PH1 and PH2 are most similar to the PH domain in *Dictyostelium* protein kinase B homologs, but the C-terminal half of the third PH domain (PH3) is not well conserved and lacks significant homology to other known PH domains. Sequence analysis (Fig. 3C) indicates that myosin-X PH2 and PH3 contain several residues conserved in other proteins that bind phosphatidylinositol lipids phosphorylated at the 3-position through the action of phosphatidylinositol-3-kinase (PI3K) (Isakoff et al., 1998; Rameh et al., 1997).

#### A MyTH4 and a FERM domain similar to several different myosins and a kinesin

The myosin-X tail also contains an intriguing region of approximately 150 aa (the myosin tail homology 4, or MyTH4 domain) that shares similarity with the tail domains of myosins from several other classes and a plant kinesin protein. The core conserved region of the myosin-X MyTH4 domain is ~26% identical to other MyTH4 domains such as those found in myosin-VIIa (Chen et al., 1996) and myosin-XV (Wang et al., 1998), both of which are associated with hereditary deafness in humans and mice. Remarkably, the kinesin-like calmodulin binding protein (Reddy et al., 1996), a microtubule-based

motor from plants, also contains a MyTH4 domain. A full alignment of MyTH4 domains has been published recently (Liang et al., 1999).

The carboxyl terminal end of the myosin-X tail contains a region of 250-300 aa known as the FERM domain (Chishti et al., 1998), found in Band 4.1-like proteins and the N-terminal portion of ezrin/radixin/moesin. Although FERM domains exhibit relatively low overall protein sequence identity with one another (roughly 20-50% identity between different protein families), these domains have been identified in a large number of proteins, including talin, merlin (the neurofibromatosis 2 gene), and certain tyrosine phosphatases. Other motor proteins with FERM domains include myosin-VIIa, myosin-XV, and the kinesin-like calmodulin binding protein.

## Myosin-X mRNA is widely expressed in vertebrate tissues

In order to determine the tissue distribution of this novel myosin, we probed a human multiple tissue northern blot and human master blot under stringent hybridization conditions with a 1 kb segment of human myosin-X cDNA corresponding largely to 3′ untranslated sequence (Fig. 4). By northern analysis, human myosin-X is widely distributed and has a primary mRNA transcript of approx. 9.2 kb in most tissues (Fig. 4A). Interestingly, the predominant transcript in human brain was only 6.0-6.9 kb. A northern blot from rat demonstrated that the myosin-X transcript in rat brain was also smaller than in other tissues (not shown). The human RNA dot blot (Fig. 4B) indicates that myosin-X is nearly ubiquitous in the represented tissues. The same samples, when probed for myosin-Va, resulted in a dramatically different distribution (O. Rodriguez, personal communication) and when probed for ubiquitin demonstrated equal loading of all samples (not shown). High levels of myosin-X mRNA expression were detected in kidney, testis, ovary, pancreas, pituitary gland, thyroid gland, liver, lung, stomach, small intestine, colon, placenta, and most regions of the brain. Moderate levels of myosin-X expression were detected in prostate, adrenal gland, salivary gland, mammary gland, spleen, and trachea. Myosin-X message was relatively low in muscular tissues such as heart, aorta, skeletal muscle, bladder, and uterus; expression was also limited in hematopoietic/immune tissues such as bone marrow, thymus, peripheral leukocytes, and lymph nodes. While these data suggest that myosin-X expression is relatively higher in some tissues than in others, it is clear that myosin-X mRNA is present to some degree in virtually all tissues sampled.

To examine which cell types express myosin-X mRNA, we performed in situ hybridizations on adult mouse brain and testis sections using mouse myosin-X antisense and control sense RNA probes (Fig. 5). The most intense staining in brain was detected in cerebellar Purkinje cells, where myosin-X mRNA was localized to the cell body and proximal dendrites. In testis, myosin-X staining was most intense in a circumferential distribution around the basal aspect of the seminiferous tubule. This staining pattern is consistent with localization in Sertoli cells, which are supporting cells that partially envelop the developing germ cells by way of a specialized cell-cell junction known as the ectoplasmic specialization, which consists of microtubules, endoplasmic reticulum, and f-actin (Grove and Vogl, 1989).

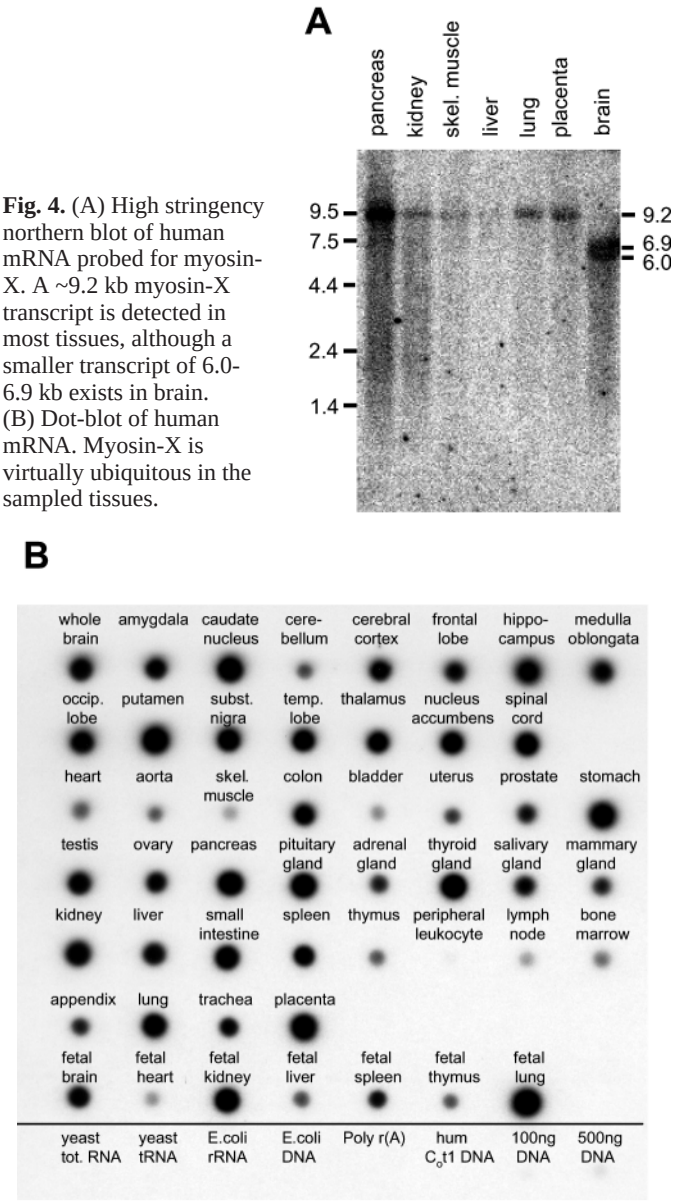


**Fig. 4.** (A) High stringency northern blot of human mRNA probed for myosin-X. A ~9.2 kb myosin-X transcript is detected in most tissues, although a smaller transcript of 6.0-6.9 kb exists in brain. (B) Dot-blot of human mRNA. Myosin-X is virtually ubiquitous in the sampled tissues.

## Myosin-X protein expression in tissues and cells

Since nothing was previously known about the distribution and biochemical properties of class X myosins, we raised specific antibodies against the head and tail of myosin-X (Fig. 1C). In immunoblots of cultured MDBK cells, both antibodies detect a ~235 kDa band (Fig. 6A, lanes 1 and 2) that is clearly of higher molecular mass than myosin-II (Fig. 6A, lane 3), suggesting that this band is indeed the full-length myosin-X. In bovine tissues, myosin-X antibodies detect a ~235 kDa band that appears to be most abundant in kidney and testis, less abundant in liver, and below the detection threshold in lung (Fig. 6B). Note that in bovine brain, antibodies to the myosin-X head (Fig. 6B, lanes 5 and 6) or tail (Fig. 6B, lanes 7 and 8) both detect a smaller band of ~180 kDa, indicating that the smaller mRNA transcript detected in brain northern blots is likely to be an alternate splice form that encodes a truncated protein. Interestingly, cerebellum appears to express both full-length and smaller myosin-X isoforms, while other regions of the brain express primarily the ~180 kDa form.

To assess the approximate abundance of myosin-X, we used the ~130 kDa baculovirus-expressed head/neck/coiled coil fragment as a standard for semi-quantitative immunoblotting. This leads to the estimate that myosin-X comprises ~0.0005% of total kidney protein (not shown). In comparison, myosin-Va is thought to represent ~0.1-0.5% of the protein in brain. Similar experiments in confluent cultures of MDBK cells led to the estimate that MDBK cells contain several hundred molecules of myosin-X per cell. It is important to note, however, that sub-confluent MDBK cultures contain cells that appear 5- or 10-fold brighter than others by immunofluorescence (see below), so the actual number of myosin-X molecules may be higher in some cells than others. Nevertheless, although myosin-X is widely expressed in a variety of vertebrate tissues, it is clearly less abundant than myosin-II, myosin-V, or myosin-VI.

### Subcellular fractionation indicates that myosin-X exists in both soluble and insoluble pools

An obvious question is whether myosin-X is soluble or associated with insoluble structures such the cytoskeleton, plasma membrane, or organelles. In subcellular fractionation experiments with kidney, much of the myosin-X pelleted at 10,000 ***g***, although a significant portion remained soluble at 100,000 ***g*** (Fig. 6C). Whereas addition of ATP or latrunculin to kidney homogenates did not solubilize additional myosin-X, ATP in combination with nonionic detergents such as Triton X-100 or CHAPS increased the solubility of myosin-X (not shown). Thus, the insoluble pool of myosin-X was insensitive to treatments that disrupt interactions with the actin cytoskeleton but required additional disruption of membranes to become soluble.

### Myosin-X is a substrate for cleavage by calpain

In initial immunoblots, several bands of lower molecular mass were detected that appeared to be associated with proteolytic breakdown of myosin-X. Additionally, the presence of three PEST sites in the tail provided potential sites for cleavage by the calcium-activated protease, calpain, so we tested whether myosin-X is indeed a substrate for calpain. Kidney homogenates incubated in the absence of calpain (Fig. 6D, lanes 1-4) accumulated breakdown products over the time course studied, while kidney homogenates incubated with calpain (Fig. 6D, lanes 5-8) underwent significant proteolysis almost immediately (lane 5), resulting in an identical pattern of breakdown products. The myosin-X head antibody detected three distinct breakdown bands of approximately 123 kDa, 118 kDa and 103 kDa, which correspond well to the predicted sizes of head domain-containing fragments if myosin-X were cleaved at the three PEST sites following the coiled coil region. Other proteins such as myosin-II remained unaffected by calpain digest as visualized by immunoblotting (not shown). Thus, myosin-X is clearly an excellent substrate for calpain in vitro. These results account for the putative breakdown fragments sometimes seen on immunoblots (Fig. 6C,E) and they may explain the relatively low amount of myosin-X protein present in tissues and cells.

### Myosin-X cosediments with f-actin in an ATP-sensitive manner

One property expected of a myosin is the ability to bind f-actin in an ATP-sensitive manner. We thus performed f-actin cosedimentation assays using soluble myosin-X from cell lysates in the presence and absence of ATP (Fig. 6E). Soluble myosin-X present in a high speed supernatant from MDBK cells homogenized in the presence of 0.5 mM ATP did not significantly cosediment with f-actin. However, when the samples were treated with hexokinase and glucose to deplete the lysate of ATP (Pollard et al., 1978), the majority of myosin-X was detected in the actin pellet (lane 9), indicating that myosin-X can bind to actin either directly or indirectly in an ATP-sensitive fashion.

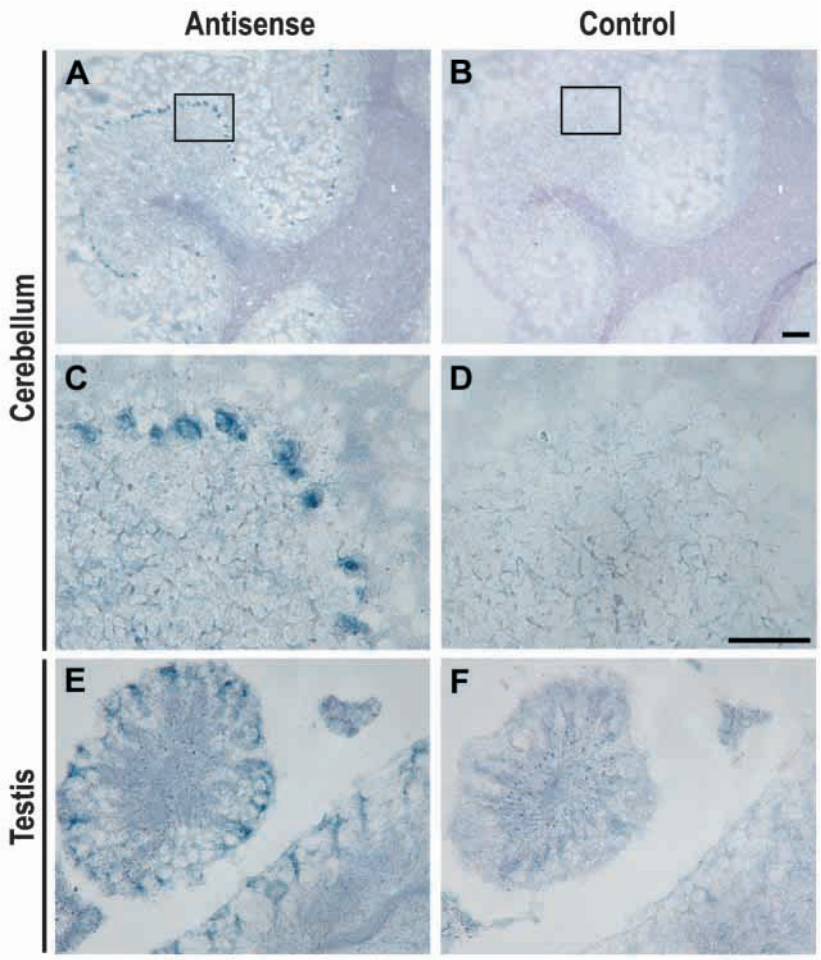


**Fig. 5.** In situ hybridization in mouse cerebellum (A-D) and testis (E-F). Antisense probe (A,C,E) detects myosin-X message in large cells of the mouse cerebellum corresponding to the characteristic pattern of Purkinje cells (A). At higher magnification, myosin-X mRNA is present throughout the cell body of the Purkinje cells (B). In mouse testis, myosin-X is detected around the perimeter of the testicular cords with occasional extension toward the lumen (E), characteristic of the Sertoli cells that surround and nurture developing germ cells. Sense control probe (B,D,F) confirms the specificity of the localization. Bars, 100 μm (A,B); 50 μm (C-F).

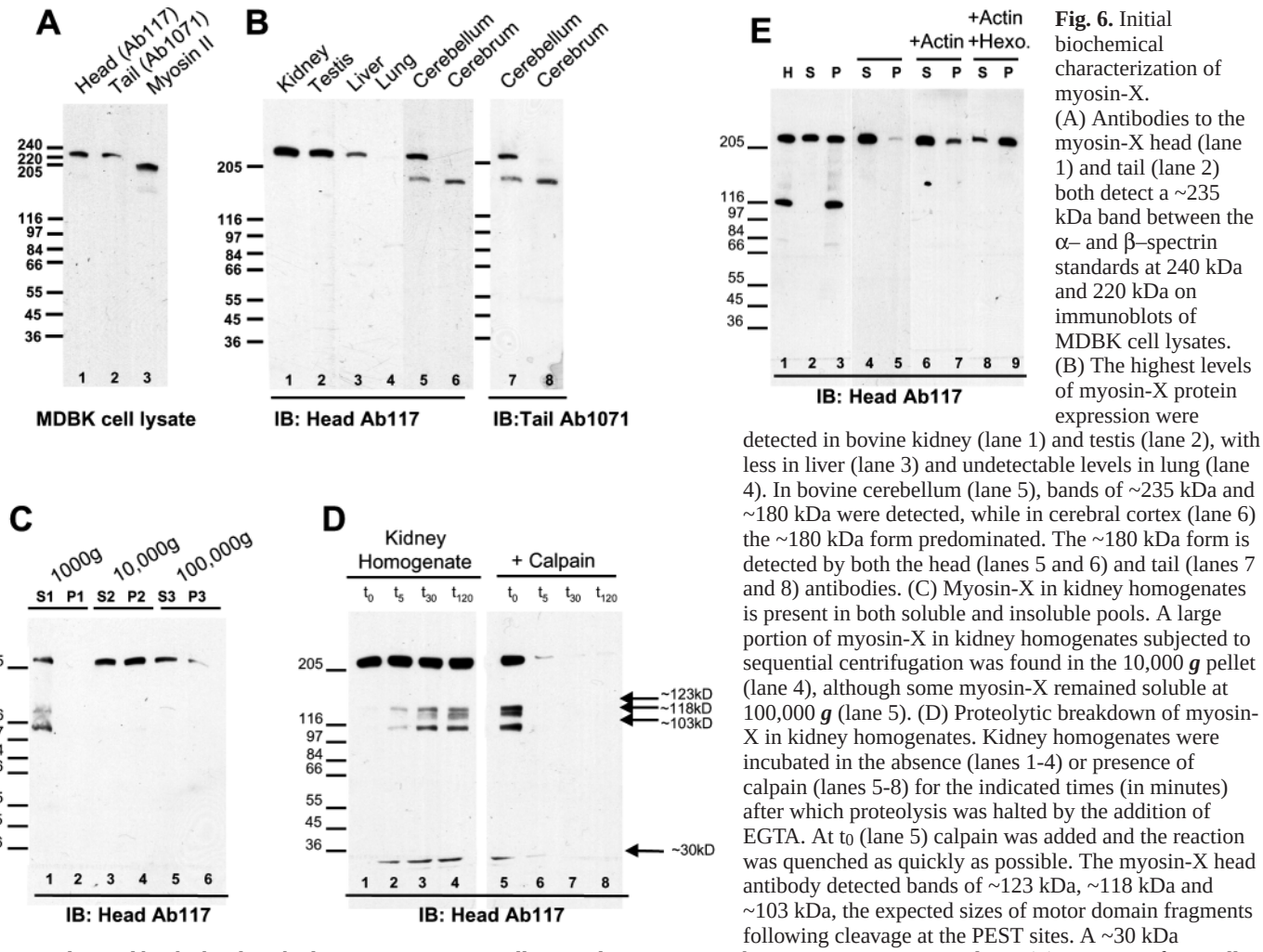


**Fig. 6.** Initial biochemical characterization of myosin-X. (A) Antibodies to the myosin-X head (lane 1) and tail (lane 2) both detect a ~235 kDa band between the α– and β–spectrin standards at 240 kDa and 220 kDa on immunoblots of MDBK cell lysates. (B) The highest levels of myosin-X protein expression were detected in bovine kidney (lane 1) and testis (lane 2), with less in liver (lane 3) and undetectable levels in lung (lane 4). In bovine cerebellum (lane 5), bands of ~235 kDa and ~180 kDa were detected, while in cerebral cortex (lane 6) the ~180 kDa form predominated. The ~180 kDa form is detected by both the head (lanes 5 and 6) and tail (lanes 7 and 8) antibodies. (C) Myosin-X in kidney homogenates is present in both soluble and insoluble pools. A large portion of myosin-X in kidney homogenates subjected to sequential centrifugation was found in the 10,000 ***g*** pellet (lane 4), although some myosin-X remained soluble at 100,000 ***g*** (lane 5). (D) Proteolytic breakdown of myosin-X in kidney homogenates. Kidney homogenates were incubated in the absence (lanes 1-4) or presence of calpain (lanes 5-8) for the indicated times (in minutes) after which proteolysis was halted by the addition of EGTA. At $t_0$ (lane 5) calpain was added and the reaction was quenched as quickly as possible. The myosin-X head antibody detected bands of ~123 kDa, ~118 kDa and ~103 kDa, the expected sizes of motor domain fragments following cleavage at the PEST sites. A ~30 kDa fragment detected by the head antibody may represent a small motor domain antigen that was resistant to proteolysis. (E) Myosin-X from cell lysates cosediments with actin. The 150,000 ***g*** supernatant (lane 2) and pellet (lane 3) from MDBK cells homogenized in buffer containing 0.5 mM ATP each contained roughly half of the myosin-X. A portion of the supernatant was then incubated with f-actin in the absence or presence of hexokinase and glucose to deplete the ATP. Samples of untreated supernatant (lanes 4 and 5), supernatant plus actin alone (lanes 6 and 7), and supernatant plus actin, hexokinase, and glucose (lanes 8 and 9) were then centrifuged at 150,000 ***g*** to obtain a second supernatant and pellet. As expected for a myosin, a significant amount of myosin-X co-precipitated with actin after removal of ATP (lane 9).

## Myosin-X associates with regions of dynamic actin in cultured cells

To elucidate possible functions of this novel motor protein, we used MDBK cells as a model system for subcellular localization. We used a bovine cell line because both the head and tail antibodies react specifically with bovine myosin-X in immunoblots. In these cells, myosin-X protein is present in membrane ruffles (Fig. 7A-C), puncta at the edge of the lamellipodium (Fig. 7D-F) and/or intensely along the lamellipodium (Fig. 7G-I). Note, however, that much of the fluorescence intensity is also distributed in small, diffuse puncta throughout the cell, often with perinuclear concentration. Using the x-z scanning capability of the confocal microscope, it is clear that myosin-X colocalizes precisely with f-actin at the edge of the lamellipodium and within ruffles (Fig. 7H, 1-3) and that many of the myosin-X puncta are cytoplasmic while others are closer to the plasma membrane (Fig. 7G, 1-3). The nature of the cytoplasmic puncta is thus far unknown. Antibodies to the myosin-X tail also stain membrane ruffles and lamellipodia prominently with additional cytoplasmic punctate staining, highly similar to the pattern detected by the head antibody (not shown).

Cells that are brightly stained for myosin-X tend to exhibit hallmarks of motility such as actin-rich membrane ruffles. In cells with less myosin-X immunoreactivity, staining is still detected in cytoplasmic puncta and at the edge of the lamellipodium. Strikingly, myosin-X is often detected at the outer tips of spoke-like bundles of f-actin within the lamella (Fig. 7D-F). These actin bundles were not associated with vinculin staining (not shown), indicating that they are not mature stress fibers, though they may represent nascent stress fibers with early focal complexes at their tips. It is also possible that these bundles are filopodia that have not extended beyond the margin of the cell, since myosin-X immunoreactivity is also seen in relatively rare filopodial processes in MDBK cells. Intense myosin-X staining is particularly evident in subconfluent cultures, where 25-30% of MDBK cells are highly immunoreactive for myosin-X, while other cells contain

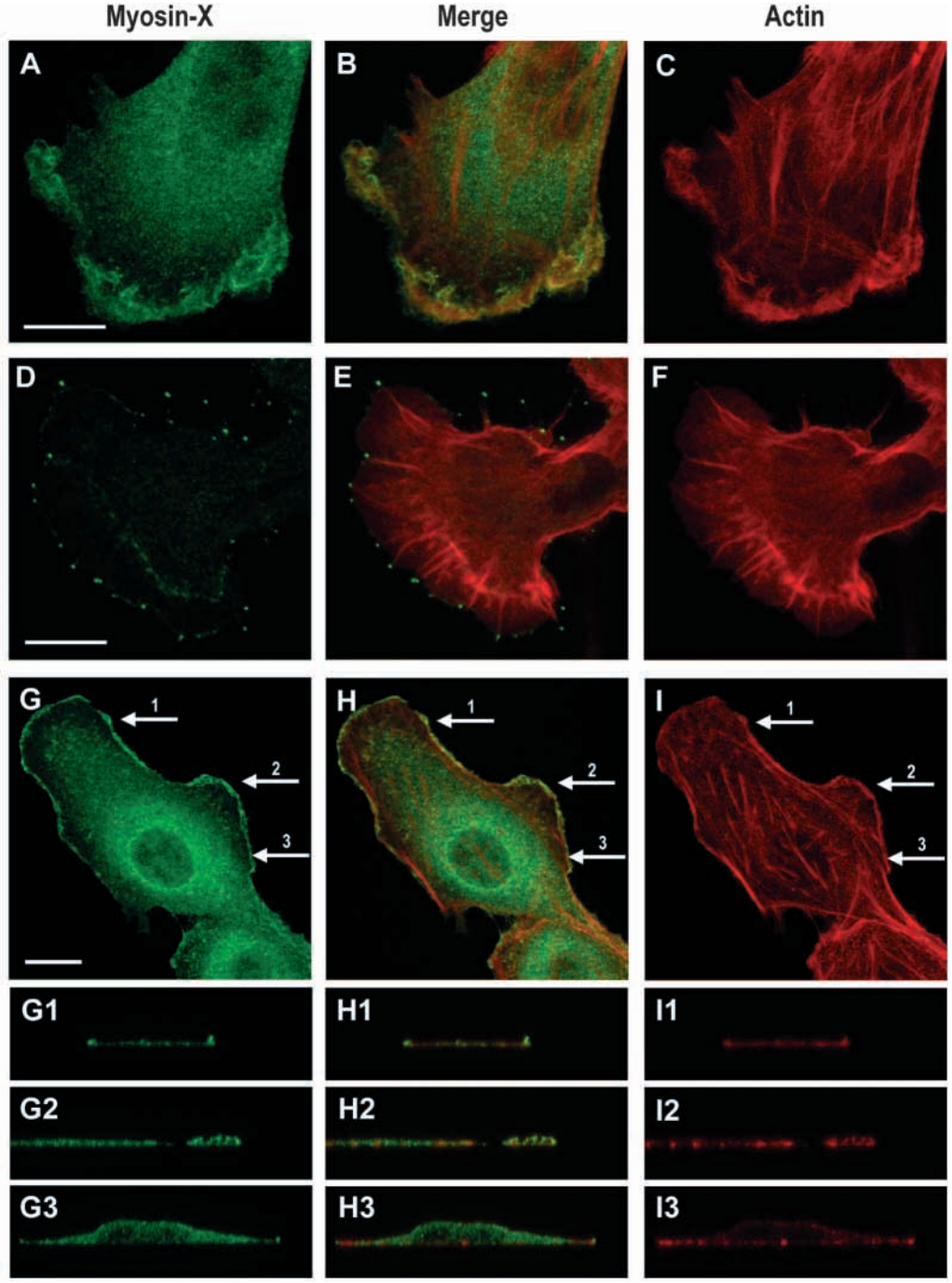


**Fig. 7.** Myosin-X associates with membrane ruffles, lamellipodia, and puncta at the tips of filopodia-like actin bundles. Specific antibodies to the myosin-X head domain were used to localize endogenous myosin-X (A,D,G) by confocal microscopy. Actin was stained using rhodamine phalloidin (C,F,I). In a brightly stained cell (A-C), myosin-X colocalized with f-actin in membrane ruffles at the leading edge. Myosin-X also localized to diffuse puncta throughout the cell. In a less immunoreactive cell without dramatic membrane ruffles (D-F), myosin-X immunoreactivity was present at the tips of spoke-like bundles of f-actin extending to the edge of the lamellipodium. In a moderately immunoreactive MDBK cell (G-I), myosin-X colocalized with f-actin along the edge of the lamellipodium (arrow 1) as well as at sites of membrane ruffling (arrow 2). Numbered arrows refer to the approximate locations of confocal x-z scans taken through the cell. Confocal x-z scans through the lamellipodium (G1,H1,I1) and a region of the cell containing membrane ruffles (G2,H2,I2) confirms that myosin-X colocalized with actin in these regions. An x-z scan through a region of the cell containing the nucleus (G3,H3,I3) indicates that the diffuse puncta of myosin-X were distributed within the cytoplasm as well as at the plasma membrane. Bars: 10 μm (A-F); 25 μm (G-I).

only background levels of staining. In highly confluent cultures, myosin-X staining intensity is lower, more uniform, and restricted largely to diffuse cytoplasmic puncta. By confocal x-z scanning, myosin-X in confluent MDBK cells is present throughout the volume of the cell, although occasionally prominent at the apical and/or basal margins, and absent from cell-cell junctions (not shown).

Although the data here illustrate myosin-X localization in MDBK cells, we have also examined other cell-types, including cow pulmonary artery endothelial (CPAE) cells, human cervical cancer (HeLa) cells, human embryonal kidney (HEK293) cells, and primary rat glia and neurons (not shown). The staining pattern in each of these cells was consistent with that seen in MDBK cells, but varied somewhat depending on the cell type. In CPAE and HeLa cells, staining of lamellipodia and the tips of filopodia-like processes was particularly prominent. In HEK293 cells myosin-X localized in cytoplasmic puncta, within filopodia, and at foci of actin around the peripery of the cell. Rat glia exhibited cytoplasmic puncta, filopodial staining, and a distribution along actin bundles within the broad lamellipodia and cell body. In primary cultured neurons, myosin-X was present in growth cones along filopodial extensions. These results confirm that the overall myosin-X staining pattern consists of several main components: diffuse cytoplasmic puncta, intense staining of membrane ruffles and lamellipodia, and distinct puncta at the

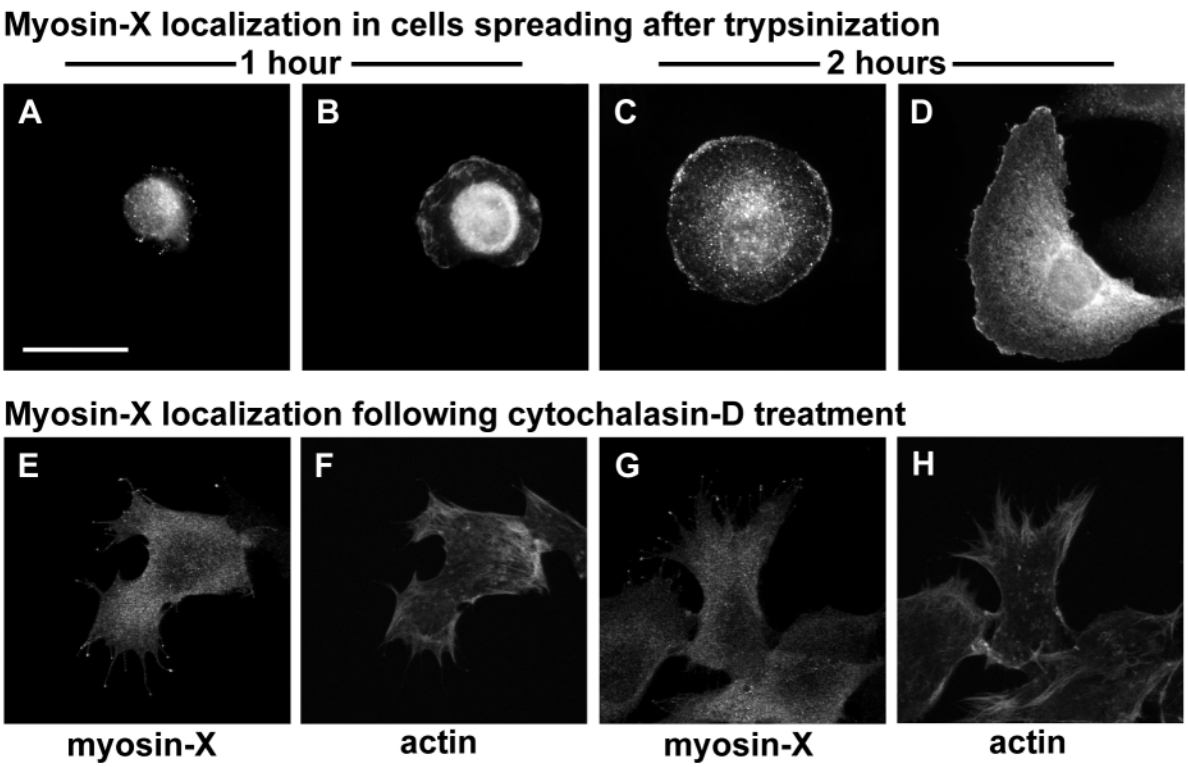


**Fig. 8.** Myosin-X localizes to regions of actin dynamics in spreading cells and remains associated with similar structures in the absence of f-actin. MDBK cells were trypsinized and replated for 1-2 hours (A-D). Representative examples of cells fixed in different stages of spreading and imaged by epifluorescence microscopy are shown. (A) Small, round cells exhibited puncta of myosin-X at the tips of processes extending from the cell body. These processes may represent filopodia or adhesive processes. (B) Myosin-X localized to membrane ruffles around the periphery and to bright puncta within the center of a spreading cell. (C) Myosin-X was associated with lamellipodia and diffuse puncta in cells of nearly full size. (D) Myosin-X staining became polarized to the leading edge in cells that appear to be undergoing motility. (E-H) Confocal extended-focus images of MDBK cells treated with cytochalasin-D exhibit myosin-X staining (E,G) at the tips of processes similar to the tips of filopodia. These structures lack a significant amount of f-actin (F,H), although some stress fibers in the cell body survive cytochalasin-D treatment. Bars, 25 μm.

tips of filopodia-like actin bundles. However, the precise staining pattern can vary within a population of cells and may depend to a certain extent on the particular morphology of different cell types.

### Myosin-X distribution is dynamic and does not rely entirely on actin

To further examine the association of myosin-X protein with regions of actin dynamics, we carried out mechanical and pharmacological perturbations of MDBK cells. Since myosin-X is largely restricted to cytoplasmic puncta in confluent cells, we wounded a confluent monolayer to determine if myosin-X redistributes to regions undergoing actin dynamics. As expected, myosin-X localized to ruffles and filopodia in cells along the edge of the wounded monolayer within 30 minutes of wounding (not shown). Next, we trypsinized and replated cells to determine the localization of myosin-X during cell spreading (Fig. 8). In small round cells, myosin-X was present in distinct puncta at the tips of processes extending from the cell body (Fig. 8A). Using confocal microscopy, we observed that these filopodia-like processes extended from all regions of the cell body, including areas not in contact with the substrate. As the cells began to spread, myosin-X was present in membrane ruffles (Fig. 8B), at the edge of lamellipodia (Fig. 8C), and later to a polarized distribution at the presumptive leading edge of the cell (Fig. 8D). Thus, it is clear that the distribution of myosin-X can be dynamic, depending on the state of motility observed in cultured cells.

Treatment of MDBK cells with cytochalasin-D (Fig. 8E-H) or latrunculin (not shown), both of which alter the actin cytoskeleton, abolished actin-rich lamellipodia and membrane ruffles and therefore myosin-X localization in such structures. In these cells, myosin-X staining was apparent in bright puncta at the tips of processes that appear to have resulted from retraction of membranes from foci of adhesion. This pattern of staining was remarkably similar to the puncta detected at the tips of actin bundles in some untreated cells (see Fig. 7D-F), and indicates that localization of myosin-X to regions of dynamic actin is not dependent solely on actin.

## DISCUSSION

We report the discovery and initial characterization of myosin-X, the founding member of a novel class of myosins characterized by a unique tail structure with three pleckstrin homology domains. Myosin-X is widely expressed in vertebrate tissues, though apparently less abundantly than several other myosins. The tail domain structure, biochemical data, and localization results that we have presented suggest a

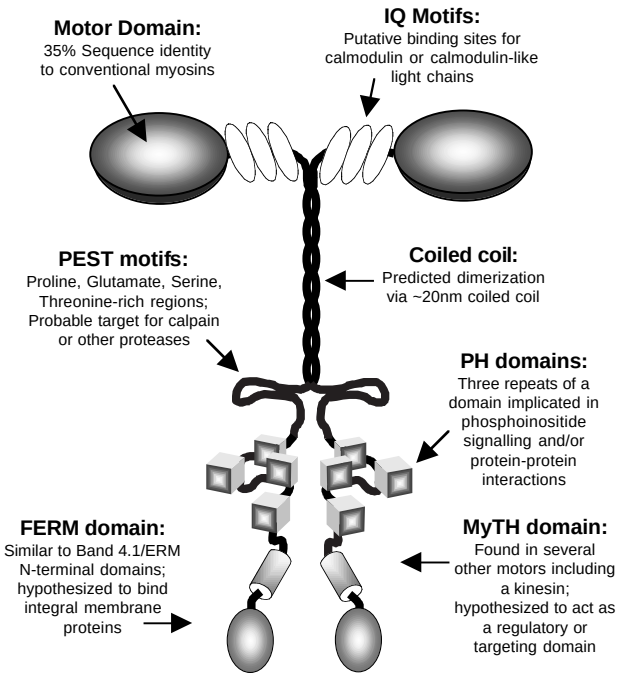


**Fig. 9.** Hypothetical model of two myosin-X heavy chains that dimerize via coiled coil interactions. This model proposes that the myosin-X dimer binds six light chains and contains ten potential sites (six PH domains, two MyTH4 domains, and two FERM domains) for interactions with proteins or membranes.

role for myosin-X in regions of dynamic actin. As illustrated in the model we propose in Fig. 9, if myosin-X exists as a dimer, as suggested by the presence of a coiled coil domain, then as many as six PH domains, two MyTH4 domains, and two FERM domains could be exposed for interactions with membrane phospholipids or other proteins, thus allowing assembly of a large molecular complex.

## Structure and function of myosin-X

Although the myosin-X motor domain is only 35% identical to the motor domain of myosin-II, conserved sequences within this region suggest that myosin-X can in fact operate as an actin-based motor. We have demonstrated that soluble myosin-X sediments with f-actin in an ATP-sensitive fashion, consistent with the properties of other myosins. In addition, recent evidence indicates that a baculovirus-expressed myosin-X head domain in fact functions as an actin-based motor (Chen et al., 1999). However, since myosin-X remains at the tips of adhesion-like processes in cells following treatment with cytochalasin-D, the interaction of myosin-X with these structures may not depend entirely on actin. Instead, the myosin-X tail domains (the PH domains, MyTH4 and/or FERM domain) could be at least partially responsible for its localization. Furthermore, the observation that myosin-X immunoreactivity varies within a population of cells is consistent with the possibility of proteolysis at the PEST sites, since we have shown that myosin-X is an excellent substrate for calpain in vitro.

Interestingly, it appears that there may be non-motor proteins whose structures would resemble a distal tail fragment of myosin-X resulting from cleavage at a PEST site. A partial cDNA sequence from human brain (KIAA1200; accession #BAA86514) is predicted to contain two PH domains, a MyTH4 domain, and a FERM domain with approx. 25% identity to myosin-X along the corresponding part of the myosin-X tail. Previously undescribed putative proteins from genomic sequences of *Drosophila melanogaster* (accession #AL031583) and *C. elegans* (accession #Z78059) also share structural similarity to the myosin-X tail and KIAA1200, despite the absence of class X myosins in these organisms. It therefore appears that the combination of PH, MyTH4, and FERM domains found in the myosin-X tail may have a novel functional relationship with each other, distinct from the identity of myosin-X as a molecular motor.

Until the discovery of the MyTH4 domain (Chen et al., 1996), it was generally believed that there was little or no similarity in the tails of myosins from different classes except for the existence of SH3 domains in certain class I and class IV myosins and the presence of coiled coil structure in many other myosins. As reviewed elsewhere, a number of unconventional myosins including myosin-X contain MyTH4 and FERM domains (Oliver et al., 1999). While the functions of these shared domains are still largely unknown, their presence in the tail domains of otherwise different motors raises the possibility of a shared system for docking and/or regulation. Biochemical evidence from other FERM domain-containing proteins indicates that this domain may be particularly important for binding to integral membrane proteins such as CD44, ICAM-1, -2, and –3, and CD43 (Tsukita and Yonemura, 1999), suggesting a mechanism by which myosin-X could be targeted to the actin-membrane interface. Consistent with this possibility, we have shown that a significant portion of myosin-X remains insoluble despite treatments that perturb the actin cytoskeleton.

Although many proteins contain one or two PH domains, myosin-X is somewhat unusual in that it contains three PH domains, one of which is split by the insertion of another. Myosin-X does not appear closely related to *Dictyostelium* MyoM, a recently discovered unconventional myosin whose tail domain contains a single PH domain and a Dbl homology domain (Schwarz et al., 1999). Although the PH domains found in MyoM and a member of the kinesin family of microtubule motors, Unc104/Kif1a (Ponting, 1995), are not closely related to those of myosin-X, the presence of PH domains in actin-based and microtubule-based molecular motors suggests the possibility that phospholipids may play a general role in targeting and/or regulation of motors.

Most PH domains are now thought to bind with varying degrees of specificity (Kavran et al., 1998; Rameh et al., 1997; Salim et al., 1996) to membrane phospholipids such as $PIP_2$, now well known as a regulator of the actin cytoskeleton (Toker, 1998), and $PI(3,4)P_2$ and $PI(3,4,5)P_3$, which are second messengers in phosphatidylinositol-3-kinase (PI3K) signaling pathways important in numerous facets of cellular physiology (Rameh and Cantley, 1999). Sequence analysis suggested that myosin-X PH domains 2 and 3 may be able to bind $PI(3,4)P_2$ or $PI(3,4,5)P_3$ (Fig. 3C), and importantly, a modified yeast two-hybrid assay was previously used to show that PH domain 2 from myosin-X can in fact bind to PI3K products (Isakoff et al., 1998). There is also evidence that PH domains may be involved in protein-protein interactions (Glaven et al., 1999;

Tanaka et al., 1999; Touhara et al., 1994; Yao et al., 1999), suggesting more complex targeting and regulation of proteins containing PH domains.

### Localization, biochemistry, and possible functional roles of myosin-X

Lamellipodia, membrane ruffles, and filopodia are protrusive structures associated with actin-based motility at the leading edge of motile cells (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Numerous proteins associated with cell motility or regulation of signaling have been localized to these structures, including Arp2/3 (Welch et al., 1997), coronin (Mishima and Nishida, 1999), and ARF6 (Song et al., 1998). Interestingly, class I myosins in yeast localize to regions of actin polarization (Anderson et al., 1998) and may be involved in nucleation of actin through their interactions with members of the WASP family (Evangelista et al., 2000; Lechler et al., 2000), suggesting that myosins-I may be involved in actin polymerization at the edge of the lamellipodium. Localization of myosin-X to regions of dynamic actin may implicate it as a candidate motor for similar processes at the leading edge.

Of particular relevance to myosin-X function, it has been reported that PIP2 and PI(3,4,5)P3 play signaling roles in regions of dynamic actin (Honda et al., 1999; Zhou et al., 1998), and PI3K signaling has been implicated in a number of cellular functions (Rameh and Cantley, 1999) including phagocytosis (Araki et al., 1996) and nerve growth cone guidance (Ming et al., 1999). Still to be resolved is whether the myosin-X PH domains serve to recruit myosin-X to a particular site in response to phospholipid signaling or whether other domains in the myosin-X tail target the protein to its appropriate location and phosphatidylinositol phospholipids regulate the function of myosin-X.

Although the mechanisms of actin dynamics at the cell periphery are now becoming better understood, the roles of actin-based motors in these processes are still unclear. We have identified a novel myosin, widely expressed in vertebrate tissues, which is a component of subcellular compartments associated with dynamic actin. Although the precise role of myosin-X is yet to be determined, the tail domain structure proposed to form a multi-protein/phospholipid complex, the previous evidence that PH domain 2 binds to products of PI3K (Isakoff et al., 1998), and the localization of endogenous myosin-X to lamellipodia and filopodia clearly implicates myosin-X at the interface between the actin cytoskeleton and plasma membrane signalling in regions of active actin dynamics.

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## REFERENCES

**Anderson, B. L., Boldogh, I., Evangelista, M., Boone, C., Greene, L. A. and Pon, L. A.** (1998). The Src homology domain 3 (SH3) of a yeast type I myosin, Myo5p, binds to verprolin and is required for targeting to sites of actin polarization. *J. Cell Biol.* **141**, 1357-1370.

**Araki, N., Johnson, M. T. and Swanson, J. A.** (1996). A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J. Cell Biol.* **135**, 1249-1260.

**Bahler, M.** (2000). Are class III and class IX myosins motorized signalling molecules? *Biochim. Biophys. Acta* **1496**, 52-59.

**Baker, J. P. and Titus, M. A.** (1998). Myosins: matching functions with motors. *Curr. Opin. Cell Biol.* **10**, 80-86.

**Bateman, A., Birney, E., Durbin, R., Eddy, S. R., Howe, K. L. and Sonnhammer, E. L.** (2000). The Pfam protein families database. *Nucl. Acids Res.* **28**, 263-266.

**Bement, W. M. and Mooseker, M. S.** (1995). TEDS rule: a molecular rationale for differential regulation of myosins by phosphorylation of the heavy chain head. *Cell Motil. Cytoskel.* **31**, 87-92.

**Berger, B.** (1995). Algorithms for protein structural motif recognition. *J. Comput. Biol.* **2**, 125-138.

**Chen, Z. Y., Hasson, T., Kelley, P. M., Schwender, B. J., Schwartz, M. F., Ramakrishnan, M., Kimberling, W. J., Mooseker, M. S. and Corey, D. P.** (1996). Molecular cloning and domain structure of human myosin-VIIa, the gene product defective in Usher syndrome 1B. *Genomics* **36**, 440-448.

**Chen, L. C., Wang, F., Meng, H., Corey, D. P. and Sellers, J. R.** (1999). Baculovirus-mediated expression of myosin X. *Mol. Biol. Cell* **10S**, 164a, abstract #952.

**Cheney, R. E. and Mooseker, M. S.** (1992). Unconventional myosins. *Curr. Opin. Cell Biol.* **4**, 27-35.

**Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N. et al.** (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane [letter]. *Trends Biochem. Sci.* **23**, 281-282.

**Evangelista, M., Klebl, B. M., Tong, A. H., Webb, B. A., Leeuw, T., Leberer, E., Whiteway, M., Thomas, D. Y. and Boone, C.** (2000). A role for myosin-I in actin assembly through interactions with Vrp1p, Bee1p, and the Arp2/3 complex [see comments]. *J. Cell Biol.* **148**, 353-362.

**Devereux, J., Haeberli, P. and Smithies, O.** (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids. Res.* **12**, 387-395.

**Frangioni, J. V. and Neel, B. G.** (1993). Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal. Biochem.* **210**, 179-187.

**Glaven, J. A., Whitehead, I., Bagrodia, S., Kay, R. and Cerione, R. A.** (1999). The Dbl-related protein, Lfc, localizes to microtubules and mediates the activation of Rac signaling pathways in cells. *J. Biol. Chem.* **274**, 2279-2285.

**Grove, B. D. and Vogl, A. W.** (1989). Sertoli cell ectoplasmic specializations: a type of actin-associated adhesion junction? *J. Cell Sci.* **93**, 309-323.

**Harlow, E. and Lane, D.** (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

**Haslam, R. J., Koide, H. B. and Hemmings, B. A.** (1993). Pleckstrin domain homology [letter]. *Nature* **363**, 309-310.

**Hasson, T., Skowron, J. F., Gilbert, D. J., Avraham, K. B., Perry, W. L., Bement, W. M., Anderson, B. L., Sherr, E. H., Chen, Z. Y., Greene, L. A. et al.** (1996). Mapping of unconventional myosins in mouse and human. *Genomics* **36**, 431-439.

**Hasson, T. and Mooseker, M. S.** (1997). The growing family of myosin motors and their role in neurons and sensory cells. *Curr. Opin. Neurobiol.* **7**, 615-623.

**Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A. et al.** (1999). Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521-532.

**Isakoff, S. J., Cardozo, T., Andreev, J., Li, Z., Ferguson, K. M., Abagyan, R., Lemmon, M. A., Aronheim, A. and Skolnik, E. Y.** (1998). Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel in vivo assay in yeast. *EMBO J.* **17**, 5374-5387.

**Kavran, J. M., Klein, D. E., Lee, A., Falasca, M., Isakoff, S. J., Skolnik, E. Y. and Lemmon, M. A.** (1998). Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains. *J. Biol. Chem.* **273**, 30497-30508.

**Kozak, M.** (1986). Point mutations define a sequence flanking the AUG

initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283-292.

**Lauffenburger, D. A. and Horwitz, A. F.** (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

**Lechler, T., Shevchenko, A. and Li, R.** (2000). Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization [see comments]. *J. Cell Biol.* **148**, 363-373.

**Lemmon, M. A., Ferguson, K. M. and Schlessinger, J.** (1996). PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* **85**, 621-624.

**Li, H. S., Porter, J. A. and Montell, C.** (1998). Requirement for the NINAC kinase/myosin for stable termination of the visual cascade. *J. Neurosci.* **18**, 9601-9606.

**Liang, Y., Wang, A., Belyantseva, I. A., Anderson, D. W., Probst, F. J., Barber, T. D., Miller, W., Touchman, J. W., Jin, L., Sullivan, S. L. et al.** (1999). Characterization of the human and mouse unconventional myosin XV genes responsible for hereditary deafness DFNB3 and shaker 2. *Genomics* **61**, 243-258.

**Lupas, A., Van Dyke, M. and Stock, J.** (1991). Predicting coiled coils from protein sequences. *Science* **252**, 1162-1164.

**Macias, M. J., Musacchio, A., Ponstingl, H., Nilges, M., Saraste, M. and Oschkinat, H.** (1994). Structure of the pleckstrin homology domain from beta-spectrin. *Nature* **369**, 675-677.

**Mayer, B. J., Ren, R., Clark, K. L. and Baltimore, D.** (1993). A putative modular domain present in diverse signaling proteins [letter]. *Cell* **73**, 629-630.

**Mermall, V., Post, P. L. and Mooseker, M. S.** (1998). Unconventional myosins in cell movement, membrane traffic, and signal transduction. *Science* **279**, 527-533.

**Ming, G., Song, H., Berninger, B., Inagaki, N., Tessier-Lavigne, M. and Poo, M.** (1999). Phospholipase C-gamma and phosphoinositide 3-kinase mediate cytoplasmic signaling in nerve growth cone guidance. *Neuron* **23**, 139-148.

**Mishima, M. and Nishida, E.** (1999). Coronin localizes to leading edges and is involved in cell spreading and lamellipodium extension in vertebrate cells. *J. Cell Sci.* **112**, 2833-2842.

**Mitchison, T. J. and Cramer, L. P.** (1996). Actin-based cell motility and cell locomotion. *Cell* **84**, 371-379.

**Mooseker, M. S. and Cheney, R. E.** (1995). Unconventional myosins. *Annu. Rev. Cell Dev. Biol.* **11**, 633-675.

**Muller, R. T., Honnert, U., Reinhard, J. and Bahler, M.** (1997). The rat myosin myr 5 is a GTPase-activating protein for Rho in vivo: essential role of arginine 1695. *Mol. Biol. Cell* **8**, 2039-2053.

**Musacchio, A., Gibson, T., Rice, P., Thompson, J. and Saraste, M.** (1993). The PH domain: a common piece in the structural patchwork of signalling proteins. *Trends Biochem. Sci.* **18**, 343-348.

**Oliver, T. N., Berg, J. S. and Cheney, R. E.** (1999). Tails of unconventional myosins. *Cell. Mol. Life Sci.* **56**, 243-257.

**Overhauser, J., Huang, X., Gersh, M., Wilson, W., McMahon, J., Bengtsson, U., Rojas, K., Meyer, M. and Wasmuth, J. J.** (1994). Molecular and phenotypic mapping of the short arm of chromosome 5: sublocalization of the critical region for the cri-du-chat syndrome. *Hum. Mol. Genet.* **3**, 247-252.

**Pollard, T. D., Stafford, W. F. and Porter, M. E.** (1978). Characterization of a second myosin from Acanthamoeba castellanii. *J. Biol. Chem.* **253**, 4798-4808.

**Ponting, C. P.** (1995). AF-6/cno: neither a kinesin nor a myosin, but a bit of both. *Trends Biochem. Sci.* **20**, 265-266.

**Post, P. L., Bokoch, G. M. and Mooseker, M. S.** (1998). Human myosin-IXb is a mechanochemically active motor and a GAP for rho. *J. Cell Sci.* **111**, 941-950.

**Rameh, L. E., Arvidsson, A., Carraway, K. L., 3rd, Couvillon, A. D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M. P., Ravichandran, K. S., Burakoff, S. J. et al.** (1997). A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J. Biol. Chem.* **272**, 22059-22066.

**Rameh, L. E. and Cantley, L. C.** (1999). The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.* **274**, 8347-8350.

**Rechsteiner, M. and Rogers, S. W.** (1996). PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* **21**, 267-271.

**Reddy, A. S., Safadi, F., Narasimhulu, S. B., Golovkin, M. and Hu, X.** (1996). A novel plant calmodulin-binding protein with a kinesin heavy chain motor domain. *J. Biol. Chem.* **271**, 7052-7060.

**Rojas, K., Serrano de la Pena, L., Gallardo, T., Simmons, A., Nyce, K., McGrath, R., Considine, E., Vasko, A. J., Peterson, E., Grady, D. et al.** (1999). Physical map and characterization of transcripts in the candidate interval for familial chondrocalcinosis at chromosome 5p15.1. *Genomics* **62**, 177-183.

**Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C. et al.** (1996). Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.* **15**, 6241-6250.

**Schwarz, E. C., Geissler, H. and Soldati, T.** (1999). A potentially exhaustive screening strategy reveals two novel divergent myosins in Dictyostelium. *Cell. Biochem. Biophys.* **30**, 413-435.

**Sellers, J. R.** (2000). Myosins: a diverse superfamily. *Biochim. Biophys. Acta* **1496**, 3-22.

**Solc, C. F., Derfler, B. H., Duyk, G. M. and Corey, D. P.** (1994). Molecular cloning of myosins from the bullfrog saccular macula: A candidate for the hair-cell adaptation motor. *Auditory Neurosci.* **1**, 63-75.

**Song, J., Khachikian, Z., Radhakrishna, H. and Donaldson, J. G.** (1998). Localization of endogenous ARF6 to sites of cortical actin rearrangement and involvement of ARF6 in cell spreading. *J. Cell Sci.* **111**, 2257-2267.

**Tanaka, M., Konishi, H., Touhara, K., Sakane, F., Hirata, M., Ono, Y. and Kikkawa, U.** (1999). Identification of myosin II as a binding protein to the PH domain of protein kinase B [In Process Citation]. *Biochem. Biophys. Res. Commun.* **255**, 169-174.

**Toker, A.** (1998). The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. *Curr. Opin. Cell Biol.* **10**, 254-261.

**Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G. and Lefkowitz, R. J.** (1994). Binding of G protein beta gamma-subunits to pleckstrin homology domains. *J. Biol. Chem.* **269**, 10217-10220.

**Tsukita, S. and Yonemura, S.** (1999). Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins. *J. Biol. Chem.* **274**, 34507-34510.

**Wang, A., Liang, Y., Fridell, R. A., Probst, F. J., Wilcox, E. R., Touchman, J. W., Morton, C. C., Morell, R. J., Noben-Trauth, K., Camper, S. A. et al.** (1998). Association of unconventional myosin MYO15 mutations with human nonsyndromic deafness DFNB3 [see comments]. *Science* **280**, 1447-1451.

**Welch, M. D., DePace, A. H., Verma, S., Iwamatsu, A. and Mitchison, T. J.** (1997). The human Arp2/3 complex is composed of evolutionarily conserved subunits and is localized to cellular regions of dynamic actin filament assembly. *J. Cell Biol.* **138**, 375-384.

**Yao, L., Janmey, P., Frigeri, L. G., Han, W., Fujita, J., Kawakami, Y., Apgar, J. R. and Kawakami, T.** (1999). Pleckstrin homology domains interact with filamentous actin. *J. Biol. Chem.* **274**, 19752-19761.

**Yonezawa, S., Kimura, A., Koshiba, S., Masaki, S., Ono, T., Hanai, A., Sonta, S., Kageyama, T., Takahashi, T. and Moriyama, A.** (2000). Mouse myosin X: molecular architecture and tissue expression as revealed by northern blot and in situ hybridization analyses. *Biochem. Biophys. Res. Commun.* **271**, 526-533.

**Zhou, K., Pandol, S., Bokoch, G. and Traynor-Kaplan, A. E.** (1998). Disruption of Dictyostelium PI3K genes reduces [$^{32}$P]phosphatidylinositol 3,4 bisphosphate and [$^{32}$P]phosphatidylinositol trisphosphate levels, alters F-actin distribution and impairs pinocytosis. *J. Cell Sci.* **111**, 283-294.