

Porcine Myosin-VI: Characterization of a New Mammalian Unconventional Myosin

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Abstract. We have cloned a new mammalian unconventional myosin, porcine myosin-VI from the proximal tubule cell line, LLC-PK₁ (CL4). Porcine myosin-VI is highly homologous to *Drosophila* 95F myosin heavy chain, and together these two myosins comprise a sixth class of myosin motors. Myosin-VI exhibits ATP-sensitive actin-binding activities characteristic of myosins, and it is associated with a calmodulin light chain. Within LLC-PK₁ cells, myosin-VI is soluble and does not associate with the major actin-containing domains. Within the kidney, however, myosin-VI is associated with sedimentable structures and specifically locates to the actin- and membrane-rich apical brush border domain of the

proximal tubule cells. This motor was not enriched within the glomerulus, capillaries, or distal tubules. Myosin-VI associates with the proximal tubule cytoskeleton in an ATP-sensitive fashion, suggesting that this motor is associated with the actin cytoskeleton within the proximal tubule cells. Given the difference in association of myosin-VI with the apical cytoskeleton between LLC-PK₁ cells and adult kidney, it is likely that this cell line does not fully differentiate to form functional proximal tubule cells. Myosin-VI may require the presence of additional elements, only found in vivo in proximal tubule cells, to properly locate to the apical domain.

UNCONVENTIONAL myosins have been characterized in numerous cell types as players in the actin-dependent movements of membrane compartments within the cell (reviewed in Mooseker, 1993; Pollard et al., 1991). To date, nine classes of myosins have been identified (Cheney et al., 1993b; Goodson and Spudich, 1993; Bement et al., 1994a). These classes were separated based on differences in their myosin motor domains, but each class also has a characteristic and distinct tail domain. It is thought that the tail domain is what defines the "cargo" specifically associated with each motor.

We were interested in identifying novel myosins expressed within the proximal tubule cells of the kidney. This cell type is highly polarized, and it has an actin-rich apical brush border. This brush border is similar to that seen in intestinal epithelia, and it contains known microvillar components such as villin and fimbrin, as well as terminal web proteins such as myosin-II and fodrin (Rodman et al., 1986). The proximal tubule is also highly endocytic (Christensen, 1982). The apical invaginations between the microvilli are rich in clathrin, and the subapical region, just below the microvilli, is rich in membrane vesicles (Rodman et al., 1984). We postulated that such an actin- and membrane-rich environment would contain multiple unconventional myosin species.

Using PCR technology, we undertook a characterization of the myosin isoforms expressed within the proximal tubule cells (Bement et al., 1994a). As a model for the proximal tubule, we used the porcine kidney cell line, LLC-PK₁, clone 4 (Hull et al., 1976). This cell line, upon cell-cell contact, differentiates to form a polarized monolayer with an apical brush border. This apical membrane contains a number of enzymes and sugar transport systems characteristic of the proximal tubule, and as a result, this cell line has been used extensively as a proximal tubule model (reviewed in Handler, 1986). RNA isolated from differentiating LLC-PK₁ cells was reverse transcribed and amplified by PCR using degenerate primers directed against highly conserved regions of the myosin motor domain. As a result of this PCR screen, nine different myosin-like clones were identified (Bement et al., 1994a). These clones included two conventional myosins-II, consistent with the observation that myosin-II is expressed within the terminal web of the LLC-PK₁ cell (Temm-Grove et al., 1992). The remaining unconventional myosin clones included members from five different myosin classes. Here, we characterize one of these myosin clones, an unconventional myosin called porcine myosin-VI.

The *Drosophila* 95F myosin heavy chain (MHC)¹ protein was the first example of a myosin-VI class motor (95F MHC; Kellerman and Miller, 1992). This protein was identified as a 140-kD component of fly embryo extracts that bound to

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1. Abbreviation used in this paper: MHC, myosin heavy chain.

F-actin affinity columns (Miller et al., 1989). Upon cloning and sequencing, it was found that this protein had an NH₂-terminal domain homologous to other known myosins. The tail domain of 95F MHC is unique, however, and it is alternatively spliced to yield motors with three related tail domains. Immunofluorescence localization of 95F MHC showed a punctate pattern throughout the cytoplasm of the fly embryo at all stages. It was proposed that 95F MHC served a function in actin-based vesicular movements within the developing fly.

We have cloned and characterized a mammalian homologue to 95F MHC. Porcine myosin-VI is a ubiquitous ATP-sensitive actin-binding protein associated with a calmodulin light chain. Within LLC-PK₁ cells, myosin-VI is soluble and does not associate with actin. Within the kidney, however, myosin-VI specifically locates to the actin-rich and membrane-rich apical brush border domain of the proximal tubule cells.

Materials and Methods

Cell Culture

LLC-PK₁ (clone 4) cells were generously provided by Dr. C. Slayman (Yale Medical School). The cells were maintained at subconfluence in α -MEM media (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 2% glutamine, and 100 U penicillin-streptomycin-fungizone (JRH Biosciences, Lenexa, KS). For immunofluorescence experiments, the cells were split onto acid-washed coverslips 1 or 13 d before use.

Cloning and Sequencing of Myosin-VI

A 130-bp PCR product identified as a putative LLC-PK₁ myosin clone (GenBank accession no. L29132; Bement et al., 1994a) was ³²P-labeled and used to screen a λ ZAP cDNA library prepared from LLC-PK₁ cells (provided by Dr. Robert Reilly, Yale Medical School; Haggerty et al., 1988). This screen yielded 14 overlapping partial cDNAs. Two of these cDNAs, 3B and 6C, were subcloned and sequenced yielding nucleotides 17-2920 of the myosin-VI cDNA. To isolate the 5' sequence, nested oligonucleotide primers antisense to nucleotides 135-154 (primer H1) and 273-290 (primer H2) were created. Using the cDNA library as a substrate, PCR reactions were undertaken pairing primer H2 with sense primers to the T3 or T7 promoters that flank the insert site. The products from this amplification were tested by PCR using primer H1 and the appropriate flanking primer. In this manner, a PCR product representing nucleotides (-12)-290 was isolated, and, once cloned, was used to screen the LLC-PK₁ λ ZAP library to isolate the larger cDNA clone. Five new clones containing additional 5' sequence were isolated, and one clone, no. 23, was subcloned and sequenced, yielding the first 120 nt of the myosin-VI cDNA. To isolate 3' sequence, the 3' proximal Nsi-R1 fragment from clone 3B was used to reprobe the same λ ZAP cDNA library. 87 positive clones were isolated, and one clone, no. 79, was subcloned and sequenced. cDNA no. 79 overlapped with clones 3B and 6C and provided nucleotides 2921-4504 of the total cDNA. In all cases, plasmid DNA was isolated as described by Ausubel et al. (1989) and sequenced with the 7-deaza Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, OH) as recommended by the supplier. Both strands of the entire cDNA were sequenced. DNA sequence was entered into the computer using the DNA Inspector IIe program (Textco, Inc., West Lebanon, NH). Comparisons were done using the Bestfit program from the Wisconsin Package (Devereaux et al., 1984).

Antibody Production

A 430-bp KpnI-NcoI fragment (nt 3147-3800) encoding the novel COOH-terminal tail of myosin-VI was filled in with T4 DNA polymerase and cloned into SmaI cut pGEX2-T (Amrad Corporation, Melbourne, Australia). This clone was grown in XLI-Blue bacteria, and upon induction with 0.2 mM isopropyl- β -D-thiogalactopyranoside, produced a 47-kD glutathione-S-transferase-myosin-VI tail fusion protein. This fusion protein was soluble, and it was purified as described by Smith and Johnson (1988) using a

glutathione-Sepharose column. The purified protein was concentrated and injected into rabbits. To affinity purify the rabbit serum, the same KpnI-NcoI fragment was cloned into pQE30 (QIAGEN Inc., Chatsworth, CA). The resultant His-tagged myosin-VI tail fusions were insoluble, and they were purified as described by the manufacturer over a nickel-Sepharose column (QIAGEN Inc.). The His-tagged myosin-VI fusions were dialyzed into borate buffer and coupled to cyanogen bromide-activated Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as described by the manufacturer. Rabbit serum was diluted twofold with phosphate-buffered saline and applied to the myosin-VI tail affinity column. Bound antibody was eluted with low pH, and was dialyzed into PBS. Affinity-purified antibody was stabilized by the addition of bovine serum albumin (fraction V) to 0.1 μ g/ml and sodium azide to 0.02%.

Northern Blots

Total RNA was isolated from LLC-PK₁ (clone 4) 4 d after confluence using the guanidinium lysis-CsCl gradient purification technique (Ausubel et al., 1989). 10 μ g RNA was loaded per lane on formaldehyde agarose gels and blotted onto Nytran membrane (Schleicher & Schuell, Inc., Keene, NH) as described in Ausubel et al. (1989). Strips were hybridized using ³²P-labeled restriction DNA fragments prepared using a random priming kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The "head" probe used for Northern analysis was a 900-bp ClaI-SacI fragment encompassing the 5' end of the cDNA. Identical results were obtained using a 130-bp fragment encompassing just the 5' untranslated sequence and a 130-bp head fragment, underlined in Fig. 1, which was initially isolated by PCR encoding the ATP-binding site. The "tail" probe used for Northern analysis was a 575-bp SacI fragment encompassing the COOH-terminal 156 aa of myosin-VI.

Protein Blots

A freshly slaughtered pig was purchased from a local abattoir, dissected, and its tissues were frozen immediately in liquid nitrogen. Tissues were disrupted using a dounce homogenizer in 0.1 g/2 ml homogenization buffer (10 mM imidazole, pH 6.9, 4 mM K-EDTA, 1 mM K-EGTA, 0.02% sodium azide, 0.2 mM PMSF, 5 μ g/ml aprotinin, 5 μ g/ml pepstatin, 4.5 μ g/ml chymostatin, and 5 μ g/ml leupeptin), and they were denatured by the addition of 0.25 vol boiling SDS sample buffer. Boiled samples were stored at -70°C for later use. Purified chick brain myosin-V and chicken brush border myosin-II were purified by established methods (Mooseker et al., 1978; Cheney et al., 1993a). Protein samples were separated by electrophoresis through 5-15% SDS-PAGE gels and immunoblotted as described by Carboni et al. (1988). Anti-calmodulin blots were undertaken as described in Sacks et al. (1991). Antibodies were diluted as follows: Affinity-purified rabbit anti-myosin-VI was used at a concentration of 1 μ g/ml as a primary antibody, monoclonal anti-calmodulin antibody (Upstate Biotechnology Inc., Lake Placid, NY) was used at a concentration of 0.5 μ g/ml, rabbit anti-human platelet myosin-II (Biomedical Technologies, Inc., Stoughton, MA) antibody was used at a dilution of 1:100, and mouse monoclonal anti-chicken brush border myosin-I (clone CX-1), which reacts with many vertebrate myosins-I by immunoblot, was used as undiluted culture supernatant (Carboni et al., 1988).

Subcellular Fractionation

Confluent LLC-PK₁ cells were washed with PBS before scraping into buffer A (10 mM imidazole, pH 7.2, 75 mM KCl, 5 mM MgCl₂, 0.2 mM DTT, 1 mM K-EGTA, 0.2 mM PMSF, 5 μ g/ml aprotinin, and 1 mM Pefabloc SC) (Boehringer Mannheim Biochemicals) in the presence or absence of 10 mM ATP. Cells were disrupted by 70-80 strokes with a glass dounce (Wheaton Scientific, Millville, NJ). Four mouse kidneys were homogenized in 40 ml buffer A \pm ATP using an Omnimixer at half power for 30 s. After the initial homogenization, the whole cell lysate was spun at 480 g for 10 min, generating the low speed samples. The resultant supernatant was further spun at 12,000 g for 20 min, generating the high speed samples. The high speed supernatant was spun a final time at 100,000 g for 60 min to yield the ultra speed samples. Pellets were brought up to stoichiometric volume with the appropriate buffer, and portions of all samples were mixed with 0.25 vol boiling sample buffer before gel electrophoresis. The BCA assay was used to quantitate the protein concentration of homogenized fractions, as described by the manufacturer (Pierce Chemical Co., Rockford, IL). The relative concentration of myosin-VI was determined by comparing the immunoreactive bands observed from protein blots of the homogenized cells and known concentrations of purified myosin-VI tail fusion protein.

A

-102 CTTATTTGCCCTGGTGGTGGTATGATACAGATGTCTAAATTTCCCTTGCCTGCCATTTGAGTTTACCGAGCTGGGAGTAGTGGTAACCTACTTCCAAAATGGAGGATGGAAAAACCCGTTTGGGACACACCCCTACAGATGGATT 48
M E D G K P V W A P H P T D G F 16

49 CAGGTGGCAATATCGTGATATTTGCCCTGACAGCTTAAACAATGAACCCCTGAACCAAAAAGGCAAGACTTTTGGCTCTCAATAAACCAAGTGTCCCTGCAGAGGAGCAGTAAGAAGATGGGAAGATACTGTTCAATGATG 198
Q V G N I V D I G P D S L T I E P L N Q K G K T F L A L I N Q V F P A E E D S K K D V E D N C S L M 66

199 TATTTAAATGAAGCCACACTCCCTCCATAATA TCAAAAGTTCGATACAGTAAGACAGAAITTTATACATATTTGGCCCAACTTCTGATTTGCCGTGAAACCCATCTTTGACATTCCTAAAATCTACTCTTCAGAAAACAATAAAGTCTCAACA 348
Y L N E A T L L H N I K V R Y S K D I Y T V A N I L I A V N P Y F D I P K I Y S S E T I K S Y Q 116

349 GGAAATCTCTTGGGACCATGCCACTCAATGCTTTGCAATTCGTAAGGCTTTTGAGACATGAAAGTCTCAAGCTGAGTCACTATCATTTGTA TCTGGAAATCAGAGCTGGCAAAACGGAAATA CAAAATTTGCTCTAAGA 498
G K S L G L T M P P H V F A I A D K A F R D M K V L K L S Q S I V S G E S G A G K T E N T K F V L R 166

499 TATCTGACTGATCTGGAACCCGTCAGATATTCGATGATAGAAATTTGGAAGCTTAACCTCTCTAGAGCTTTGGAAATGCAAAAGACTGTCCGCAACAATAATGCAAGTGTATTTGGGAAATTTGTAGAAATCATTTTAAATGAA 648
Y L T E S Y G T G Q D I D D R I V E A N P L L E A F G N A K T V R N N N S S R F G K F V E I H F N E 216

649 AAGAGTTCAGTGTGGAGGATTTGCTTACATTAATCTCTAGAGAAATAGGATCTGTCTCAAGGCAAGAGGAAAGAAATATCATATCTTTTATAGGTTGTGTGCTGCTTGAAGATATAGGAAAGACTTCAATTTGAGC 798
K S S V V G G F V S H Y L L E K S R I C V Q G K E E R N Y H I F Y R L C A G A S E D I R E R L H L S 266

799 TCCCGAGATAATTTGGGTATTTAAACCGGGGCTGCATCGATATTTTGTAAACAGGAACTGACAAAAGACTTTTACAGAACCAAGAAAGTCTGAGTACCTTAAGGACAGTTCCTTGAAGACCCCTTTGTAGATGACATGGAGAC 948
S P D N F R Y L N R G C T R Y F A N K E T D K Q I L Q N R K S P E Y L K A G S L K D P L L D D H G D 316

949 TTTATGATGATGTGACAGCCATGAAAAATCGGTTGGATGATGAAAAAGCTCGACTGTTCGGGTAGTACTGGCTCTCATCTTTGAAATATTTGATTTTGAGGAGCTGGCAGACTTCAGGTTGGTTTAACTGAAAGAT 1098
F I R M C T A M K K I G L D D E E K L D L F R V V A G V L H L G N I D F E E A G S T S G G C N L K N 366

1099 AAATCTACTCAGGACTTGAATATTTGTCAGAAAAATTAATCGGTTTGGATCAAGCAGTCTCTGTTAAGTTTAACTCAAGAGCTGATCAACACAGCAGGGGGCCCAAGGAAAGTATTAAGAAAGTCCCTTGAAGAGTGGAGCAA 1249
K S T Q L E G L Q D L L G L D D L R V L S L T T R V M L T T A G G A K G T V I K V P L K V E Q 416

1249 GCAAAACAATGCCCGGATGCCCTGGCAAGACTGCTATAGCCATCTTTTGTATGATGATGAAACAGATACTGTTTCTTTTGAACCTCACTATTTTATGGAGTCTGCACATTTGCTGTTTGTAGTACTTTGAACAT 1398
A N N A R D A L A K T V Y S H L F D H V V N R V N Q C F P F E T S S Y F I G V L D I A G F E Y F E H 466

1399 AACAGTTTGAACAATTTTGCATCAACTTGCACGAAAGCTTCAACAGTCTTTTAAATGAAAGTTCGAAAGGAGGACAGAACTCTATCAAAAGGAGGTTAGGTTGTAAGTAATGATTAAGTAACTGAAATGAGTATA 1548
N S F E Q F C I N Y C N E K L Q Q F P N E R I L K E E Q E L G L G V N E A H Y V D N Q D C I 516

1549 GATTTAAITGAAGCAGATTAAGTGGAAATTCGATATTTGATGAAAGAAATCGCTTCCACAGCCAGGATCAACACTTTCATCTGCAGCACCAGCAAGCAATTTCCGACTCTATTTCAAGAAATCAAGAAATCAAGCTG 1698
D L I E A R L V G I C L D I L D I L D E E N R L P Q P S D Q H F T S A G H Q K H K D H F R L S I P R K S K L 566

1699 GCAATCCATAGGAACATAGCATATGACGAAGGTTTCATATCAGGCAATTTGCAAGGGGCGATTTGCTATGAACTACTCAGTTCGTGGAAAAAATATGATGCTTTGATATGCTCTCGAGTCTTAAATGTAATCCAGGGATAA 1848
A I H R N I A Y D E G F I I R H F A G A V C Y E T T Q F V E K N N D A L H M S L E S L I C E S R D K 616

1849 TTCATCCGGGAATTTTGAATCTCCCAAAATCAACAAAGATCAACAAAAGCAGGAAACTTAGCTTTCATGACGAAACAAAGTTTAAAGACACAGTAAATTTGCTTCTGGATAAATTCGAAATTCGGAAGCAAGCTTT 1998
F I R E L F E S T N N K D T X Q K A G A A L S T V G N K F K T Q L N L L L D K L R S T G A S F 666

1999 ATTGCTGTATCAAACTAATTTAAAGATCAAGCCACCACTTTGAAGTGTCTCAGATTTTGTCTCAACTTCACTTCAGTATGTTGTTGAGTATGTTTGGACATGATGCGGGGGGTTTCACTACGGGCTTCAATTTCAAGATGTAC 2148
I R C I K P N L K M T S H H F E G A Q I L S Q L Q C S G M V S V L D L M Q G G F P S R A S F H E V 716

2149 AACATGATAAAGACTCTCCCGGATTAAGCTTGAAGATAGACCCAGACTATTTGTAAGGCTTTTAAAGCCCTGGGCTTAAATGAAATGACTACAAGTTGGGTTAAACAAAGTATTTTGAACCTGGCAAGTTTTCAGAA 2298
N M Y K K S L P D K L A R L D P R L F C K A L F K A L G L N E I D Y K F G L T K V F F R P G K F A E 766

2299 TTTGATCAGATTAAGTTCGACCTGACCACTTAGCAGAGTGGTAAAGAGTCAACTCTGGCTTATCTGCAGTCTGCTGGAAGAAATTCAGTGGTCTGCTCTCAGTCAATTAATTTGAAAAACAAAATAAATATCGAGCTGAA 2448
F D Q I M K S L P D H L A E L V K R V N H W L I C S R W K K V Q W C S L S V I K L K N K I K Y R A E 816

2449 GCCTGCAATTAAGTCAAGAACTATTCGAAATGCTGCTTTCGAAAGGAGACACAACTCCGATGAGCCCTTGTAAAGTGGCACACTGAAAAACCGCTTGA CAATTTTAAAGTGAATGATGTCGCCCTGAAAGTGGAAACAG 2598
A C I K M Q K T I R M W L C K R R H K P R I D G L V X V G T L K K R L D K F N E V V S A L K D G K Q 866

2599 GAAATGATAAAGCTTCAAGGACCTTGAATCTCTATTTGATGCTTTAATGGCAAAATTAAGTCTACTATGATGACAGGAAACAAATTCAGAAAGATATGATGCAATTAATTAAGGCTCAGCCCTCTCTCAGTATACAGAAA 2748
E M S K Q V K D L E I S I D A L M A K I K S T M M T R E Q I Q K E Y D A L V K S S A V L L S A L Q K 916

2749 AAGAAAGCAGAAAGAGAAAGCAGAAAGGCTTCAAGAGAAATGGAAAGGAAAGAAAGCAAGTGAAGAAAGTGAACCACTGAAAGGAGGAGGAAAGGAGGAAAGGAGGAAAGGAGGAAAGGAGGAAAGGAGGAAAG 2898
K K Q Q E E E A G G E E A R R I Q E E M E K E R R E E D E Q R R R K E E E E R M K E E E R M K L E M A E A K R 966

2899 CAAGAAAGAGAGAGAGAAAGAAAGGAGATGATGAAAGGATTTCAAGGCTTGAAGTGGAGGCGCAGCTGCCCGACAGCGGGAGGAGTCCAGCAGCAGGCTTCTGAGCAGGAGCGCCGGGACCGGGAAGCTGCCCTGCG 3048
Q E E E E R K K R E D E K R I Q A E V E A Q L A R Q R E E S Q Q A V L E Q E R A D R E L A L R 1016

3049 ATTGCCAGAGGAGGAGGAGCTCACTGACGAGGCGCAGGCGCACTGGGCTGCGCAGAGCCCTGCTGTA CAAGCCACAAAGCGGCTGTGTACCAAGAAATATGATCTTAAATGGAATAATGCGAACTACGGGATACC 3198
I A Q S E A E L I S D E A Q A D P G L R R G P A V Q A T X A A A G T K K Y D L S K W K Y A E L R D T 1066

3199 ATCAATACTTCTGTGATATGAGCTCTGGCAGCTGCAGAGAAATTTCA TAGGACATAAAAGTGTATCATGCTTGGAAATGCAAGCAAGAAAGAAATGAAACAGAGCAACGTCTCAAAAGTCTGTATCTGATATGCT 3348
I N T S C I E L L A A C R E F H R R L K V Y H A W K S K N K R N T E T E Q R P K S V T D Y A 1116

3349 CAGCAGAACCCAGCAGTCCCTGCCAGGACAGGAGATCGAAATGAAACCGGACAGCGGTTCTTCCGCAATCCGTTCACTCGGCTCTGGGACAGTACAAGACCCCTCAGAAATGAAGAAAGGCTGGTGGTATGCCAATTT 3498
Q Q N P A V Q L P A R Q Q E I E M N R Q Q R F F I R S A D Q Y K D P Q N K K K G W W Y A H F 1166

3499 GATGACCGTGGATCCCGGCAAAATGGAATCTCTGCAAAAACCCATCTCTTGGCTGGTAAAGTGA CA TGGAGTGTGTGAGTGAATCTTGAAGAGCAGGCTGCAAGCGTGGAGCGTGGAGAGATTTTCCCAAGA 3648
D G P W I A R Q M E L H P D K P P I L L V A G K D D M E M C E L N L E E T G L T R K R G A E I L P R 1216

3649 CAGTGTGAAGAAATTTGGAAACGCTGTGGAGGCTCAGATCTTCAAGATGCAATTTGAGACGACAGCTAGGCCACATA TGCCACGGCCTGTCAGAACCTGTAAAGTGAAGTGTGACTAACCCTTGCAGCTGGGAGCTCTTG 3798
T F E E I W E R C G G I Q Y L Q N A I E S R Q A R P T Y A T A M L Q N L L K * 1254

3799 CCAATGCTTGGTGGTGGGAGGAGTGGCCCAAGCAATGCAATCCAGGATCCAGTATGAGATTA TGTAAACAAAGTGAACAGATTTTAAATCATGCTTTTGTAAATTTAAAGTAAATACAGTATGAAATTTGGGACCTGAAATA 3948
GCTTCTGTATCCAGCTAATCTGTTGAACTCTCATGATTTTAAATCTTTTACACTTGGGACAGTCTGAACTTCTCAATCTTTTGGCAACTGACTTAAATCTATCATCTGTTCTTGAAGAAAGATTTTAAAGAAAAA 4098
TTCATTTTCTGCTTTTAAAGATGACAGTACCAATAAAGTAAAGAAACTTATGTTTCTGACAAAATAAGGCTTAAAGTAAAGCACTGATGTTTGTATGTAAGCTTGTGAAATTTTATTCATTTTTCATTTTTCAT 4248
4249 GAAATTAATGCAATTTCCGTCAGAGTGAAGATTTTATTTCTCTCACTTTTTCAGATGTTTATTTGATGAAAGCAATTTACTGAAATTTCTGAAAGAACTTAAATGATTTGATGCTATCTGTGACAAAAGAGTACAT 4398
4399 AGTATCCGCAAGATGTTTGGTCTGTCACATGTTATGCTGTAAGAAATGTTTCAATGCAAAATGAAGAAATTCAGATCAAAAAAATGAAAAA 4504

B



Figure 1. cDNA sequencing of porcine myosin-VI. (A) Nucleotide and deduced amino acid sequence of the porcine myosin-VI cDNA. Nucleotides are numbered relative to the start of the initiation codon. Below the sequence, the deduced amino acid sequence is shown starting from the initiator methionine. The TAG stop codon is indicated by an asterisk. A possible polyadenylation signal is located at nt 4463 (AATAAA). The original PCR product used to clone the myosin-VI cDNA is underlined (GenBank accession no. L29132). This PCR product was obtained using degenerate primers encoding the conserved GESGAGKT and EAFGNAKT motifs (Bement et al., 1994a). The amino acids predicted to form a coiled-coil region are boxed (Lupas et al., 1991). The total sequence data are available from EMBL/GenBank/DBJ under accession number Z3531. (B) Schematic representation of the myosin-VI cDNA. Constructed from overlapping clones nos. 23, 3B, 6C, and 79 (see Materials and Methods), this schematic shows the restriction sites used in this study. The open reading frame is denoted by a heavier line. K, KpnI; N, NcoI; S, SacI.

Results

Pig Myosin-VI Is Highly Homologous to *Drosophila* 95F MHC

To initiate our study of the multiple unconventional myosins expressed by LLC-PK₁ cells, one of the DNA fragments identified by PCR as a putative myosin (Bement et al., 1994a) was used to screen a λ ZAP cDNA library. After hybridization screenings, a cDNA of 4.6 kb was compiled (Fig. 1). The sequence revealed an open reading frame encoding a protein of 1,254 amino acids (144.7 kD; Fig. 1).

Sequence comparison of this open reading frame to other known myosins indicated that it did, in fact, encode a myosin-like protein with an NH₂-terminal motor domain similar to other myosin heads (Fig. 2, A and B). In particular, this gene product was found to be 70.9% similar to the unconventional myosin, *Drosophila* 95F MHC (Kellerman and Miller, 1992). Phylogenetic analysis indicated that these myosins together, formed the sixth class of myosin to be identified to date (Cheney et al., 1993b), and for this reason we named this LLC-PK₁-derived myosin, porcine myosin-VI.

Porcine myosin-VI harbors characteristic ATP-binding (GESGAGKT, aa 151-158; Walker et al., 1982) and actin-binding motifs (FIRCIKPN, aa 666-673; Warrick and Spudich, 1987), and it shares many conserved residues with other myosin classes along the entire length of its motor domain (Fig. 2 B). The motor or head domains of the pig and fly myosin-VI are most highly related, being 73.2% similar and 56.1% identical, while pig myosin-VI is ~53–62% similar and 27–39% identical to myosins of other classes. Class VI myosins share a 25-aa insert in the myosin head domain not seen in other classes of myosins (pig myosin-VI aa 316-329). By comparison with the three-dimensional structure of chicken muscle myosin-II S1 subfragment (Rayment et al., 1993b), this insert may add to a surface random coil on the myosin head.

After the myosin-VI motor domain, a neck portion containing a single light chain binding site (or IQ motif) is found (boxed in Fig. 2 C). All of the known myosins contain at least one light chain binding sequence (Cheney and Mooseker, 1992), but the position of the myosin-VI IQ motif is unique. In all other myosins described to date, the light chain binding site is found immediately distal to the end of the myosin head. Instead, both myosins-VI have an ~50-aa linker between the motor domain and the single IQ motif (compare locations of light chain binding motifs in myosins-I, -II, and -V to myosins-VI in Fig. 2 C). No putative calmodulin light chain binding motifs are found within this 50-aa domain, although this region is rich in aromatic and basic residues, as is seen with other light chain binding sequences.

Both myosin-VI proteins have predicted coiled-coil regions at the proximal part of the tail as judged by the protein folding program of Lupas et al. (1991) (boxed in Fig. 1), although pig myosin-VI has a larger coiled-coil component. Of the entire myosin-VI molecule, the most highly conserved region between fly and pig is the distal portion of the tail, being 63.6% identical and 76.2% similar in this region (Fig. 2 D). This tail portion does not have any homology to any other protein in the database. Interestingly, the unique tail domain is more highly conserved between pig and fly than the motor domain.

Myosin-VI Is Ubiquitously Expressed

Northern analysis indicated that the porcine myosin-VI gene had a message size of 6.0 kb (Fig. 3 A). This was unexpected because the 4.6-kb cDNA appeared to encode the entire myosin-VI coding sequence; porcine myosin-VI was homologous with *Drosophila* 95F MHC along its entire length, including the position of the initiator methionine. cDNA probes from the 5' untranslated sequence, the head domain, and the tail domain all recognized a 6.0-kb mRNA by Northern blot (Fig. 3 A and data not shown). We sequenced two different overlapping clones that contained portions of the 5' untranslated region of porcine myosin-VI and, because of this, we are confident that the open reading frame does not continue 5' of the initiator methionine indicated in Fig. 1. There are stop codons in all frames upstream of this ATG. The nucleotide sequence around this initiation codon (CCA-AAATGG) is consistent with the eukaryotic start site consensus sequence (CC ^A/_GCCATGG; Kozak, 1984), unlike the other ATG sequences within the untranslated sequence. Furthermore, as described below, the observed molecular weight of myosin-VI is similar to its predicted size of 144.7 kD.

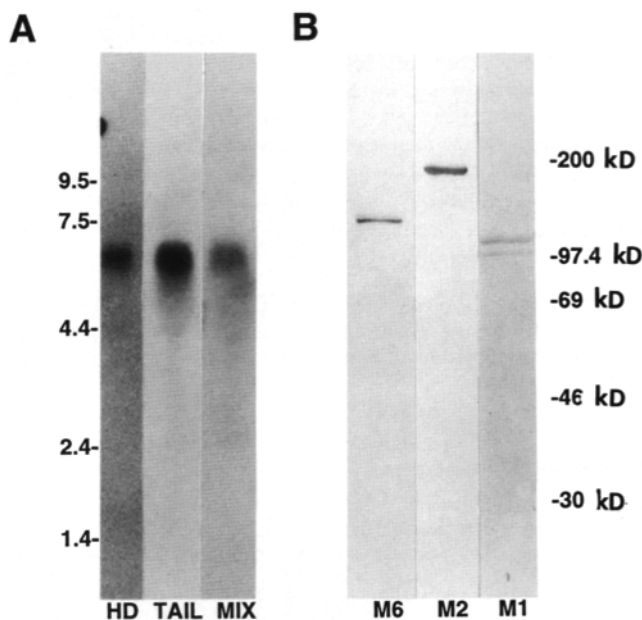


Figure 3. Expression of porcine myosin-VI in LLC-PK₁ cells. (A) Northern blot analysis using head and tail myosin-VI probes. Individual lanes containing LLC-PK₁ cell RNA were probed with either a ³²P-labeled, 900-bp NH₂-terminal SacI fragment (HD) or a 575-bp COOH-terminal SacI fragment (TAIL) or a mixture of both (MIX). All hybridizations detected a single 6-kb mRNA. The positions of molecular weight markers are shown to the left. (B) Immunoblot showing specificity of myosin-specific antibodies. Whole-cell protein from 1.8×10^5 LLC-PK₁ cells (for M6 and M2) or 5.4×10^5 LLC-PK₁ cells (for M1) was separated on a 5–16% polyacrylamide gel and transferred to nitrocellulose. Individual lanes were probed with either affinity-purified rabbit anti-myosin-VI antibodies (M6), rabbit anti-human platelet myosin-II antibodies (M2), or monoclonal anti-myosin-I antibodies (M1). The anti-myosin-I antibody recognizes head epitopes shared by many myosins-I (Carboni et al., 1988). The three antibodies detect myosins of different molecular masses: ~145 kD for myosin-VI, 200 kD for myosin-II, and 110–130 kD for myosins-I. Molecular weights are indicated to the right in kilodaltons.

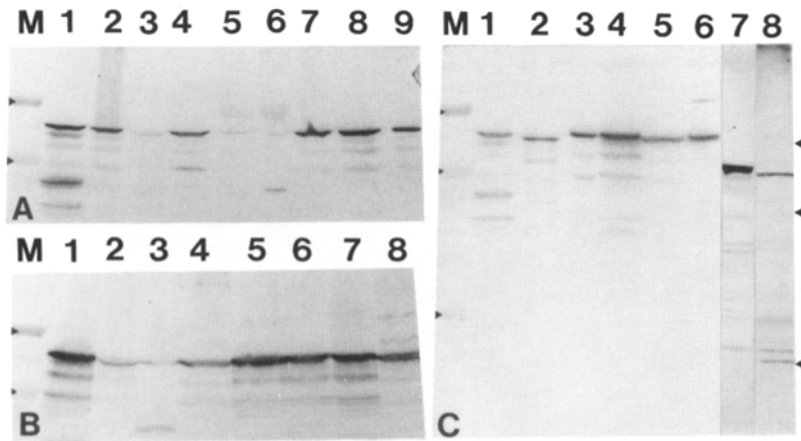


Figure 4. Myosin-VI expression in pig and rat tissues and in other organisms. (A) Detection of myosin-VI in pig tissues by immunoblotting. Total cell extracts from pig tissues were separated on a 5–16% polyacrylamide gel and blotted with the affinity-purified rabbit anti-myosin-VI antibody. Approximately equal amounts of protein were loaded in each lane for each protein source. Whole-cell extract from LLC-PK₁ cells was also included for comparison. The affinity-purified antibody recognized a 145-kD polypeptide in all tissues tested. Tissues were loaded as follows: lane 1, kidney cortex; lane 2, intestinal mucosa; lane 3, liver; lane 4, lung; lane 5, heart; lane 6, jowl muscle; lane 7, brain cortex; lane 8, brain medulla; lane 9, LLC-PK₁ (CL4). M, molecular weight markers; bullets indicate 200- and 97-kD bands.

The lower bands seen in these protein blots are attributed to myosin-VI degradation products because they are less prominent in freshly prepared samples and they increase in intensity upon storage (not shown). (B) Detection of myosin-VI in rat tissues by immunoblotting. Samples were prepared as in A. Tissues were loaded as follows: lane 1, kidney; lane 2, liver; lane 3, heart; lane 4, brain; lane 5, intestine; lane 6, stomach; lane 7, colon; lane 8, testes. Molecular weights are presented as in A. In all tissues tested, a 145-kD polypeptide was recognized. (C) Detection of myosin-VI-related proteins in other species. Tissues from varied organisms were prepared as described in A. The affinity-purified antibody recognized a 140–145-kD polypeptide in all species tested. Samples were loaded as follows: lane 1, pig kidney; lane 2, chicken intestine; lane 3, mouse kidney; lane 4, rat kidney; lane 5, the human intestinal cell line Caco-2_{BBC}; lane 6, *Xenopus* gut; lane 7, rat kidney; lane 8, *Drosophila* first instar larvae. M, molecular weight markers; bullets indicate 200-, 97-, and 43-kD bands. Lanes 7 and 8 are from a different gel than lanes 1–6.

The *Drosophila* 95F MHC mRNA is alternatively spliced yielding myosins with three different tails (Kellerman and Miller, 1992). The splicing event occurs immediately COOH-terminal to the coiled-coil domain leading to either an insert of 15 aa or a switch to a short hydrophobic tail. Exhaustive RNase protection studies indicated that porcine myosin-VI was not spliced in this region (not shown). Also, no evidence was seen for splicing events in the neck region (not shown). Alternative splicing adding additional light chain binding motifs has been observed with some mammalian myosins-I (Halsall and Hammer, 1990; Ruppert et al., 1993).

To characterize this unconventional myosin in more detail, we set out to produce myosin-VI-specific antibodies. A KpnI-NcoI DNA fragment (see Fig. 1 B) encoding the COOH-terminal 203 aa of the unique myosin-VI globular tail was cloned into pGEX-2T to produce a fusion protein containing glutathione-S-transferase at its NH₂ terminus and myosin-VI tail at its COOH terminus. Since we were interested in generating antibodies that would only recognize myosin-VI class motors, only the tail sequence unique to myosin-VI was used; sequence encoding the coiled-coil domain was not included. Antibodies were raised against this fusion protein and were affinity purified using a related myosin-VI tail fusion protein that contained only histidine residues at its NH₂ terminus. The affinity-purified antibodies were then used to characterize the expression profile of myosin-VI.

By immunoblot, myosin-VI in LLC-PK₁ cells was found to have a mobility of ~145 kD, consistent with the 144.7-kD predicted molecular weight of the open reading frame (Fig. 3B). As shown in Fig. 9 A, immunoprecipitated myosin-VI was also 145 kD. In protein blots, the mobility of myosin-VI was distinct from myosin-II (200 kD) and myosin-I immunogens (110–130 kD) observed in LLC-PK₁ extracts (Fig. 3 B). This protein blot analysis provides further evidence that the clonal LLC-PK₁ cell line does indeed express multiple

myosin-like proteins, as was suggested by the identification of multiple myosin clones by PCR.

Myosin-VI was found to be broadly expressed in all tissues tested in both pig (Fig. 4 A) and rat (Fig. 4 B), with lowest levels seen in smooth muscle and liver. We attribute the lower bands seen in these protein blots to myosin-VI degradation products because they are less prominent in freshly prepared tissues and they increase in intensity upon storage (not shown).

The affinity-purified anti-myosin-VI antibodies reacted with immunogens expressed in a wide variety of species from mammals to chickens, flies, and frogs (Fig. 4 C). In most species, the antibodies recognized a major polypeptide of ~145 kD. In humans, chickens, *Drosophila*, and *Xenopus*, the apparent mobility of myosin-VI was slightly smaller running at ~140 kD. This is the observed molecular weight for *Drosophila* 95F MHC (Miller et al., 1989). 95F MHC has a shorter coiled-coil region than porcine myosin-VI, which may account for their slight difference in molecular weight. It is unknown whether a variation in coiled-coil domain size exists in other organisms.

Myosin-VI Is Not Associated with Major F-Actin-containing Domains in LLC-PK₁ Cells

Given the ubiquitous nature of myosin-VI expression, we were interested in determining whether this myosin was generally associated with F-actin. Upon cell–cell contact, LLC-PK₁ cells form a polarized monolayer with tight junctions separating apical and basal-lateral domains. Double-labeling experiments on these polarizing cells indicated that unlike myosin-II, myosin-VI did not colocalize with major F-actin-containing structures (Fig. 5). As judged by indirect immunofluorescence, myosin-VI was not found associated with cell–cell junctions in confluent monolayers (Fig. 5, a

Myosin 6

Actin

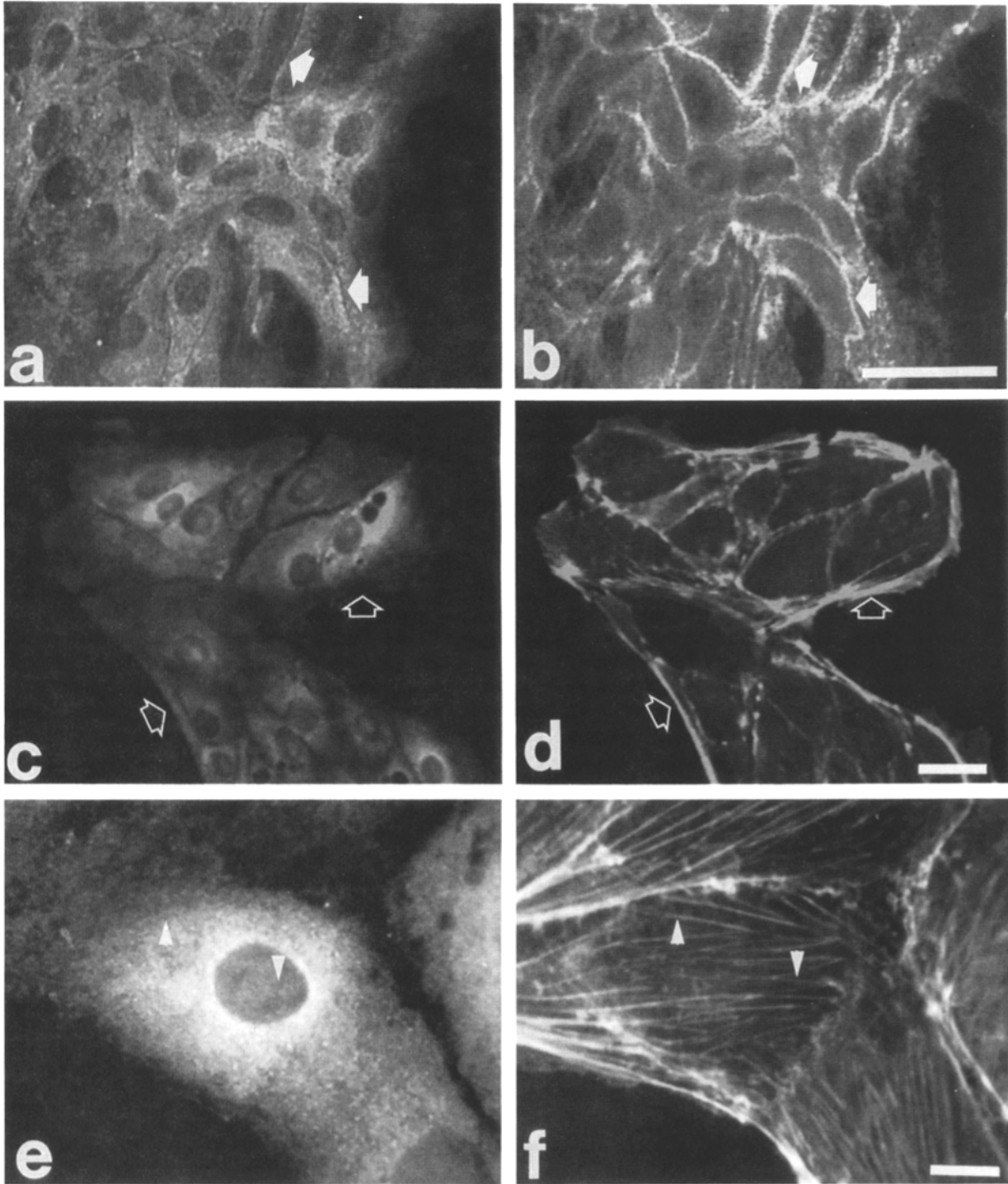
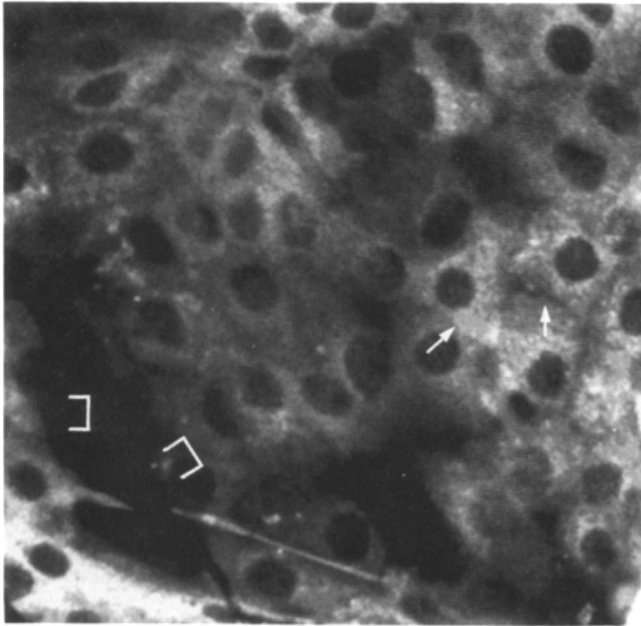


Figure 5. Localization of myosin-VI in undifferentiated LLC-PK₁ cells. Confluent (*a-b*) and subconfluent (*c-f*) LLC-PK₁ cells were grown on coverslips, fixed with paraformaldehyde, and subjected to indirect immunofluorescence with 40 $\mu\text{g/ml}$ affinity-purified rabbit anti-myosin-VI tail antibodies (*a, c, and e*) and fluorescein-phalloidin (*b, d, and f*). Cell-cell junctions are denoted by arrows (*a and b*), actin cables by open arrows (*c and d*), and stress fibers by arrowheads (*e and f*). Myosin-VI is not associated with these actin-rich structures. (*a and b*) Scanning confocal light microscopy. (*c-f*) Standard fluorescence light microscopy. Bars, 25 μm in *a-d*; 5 μm in *e and f*.

Myosin 6



Actin

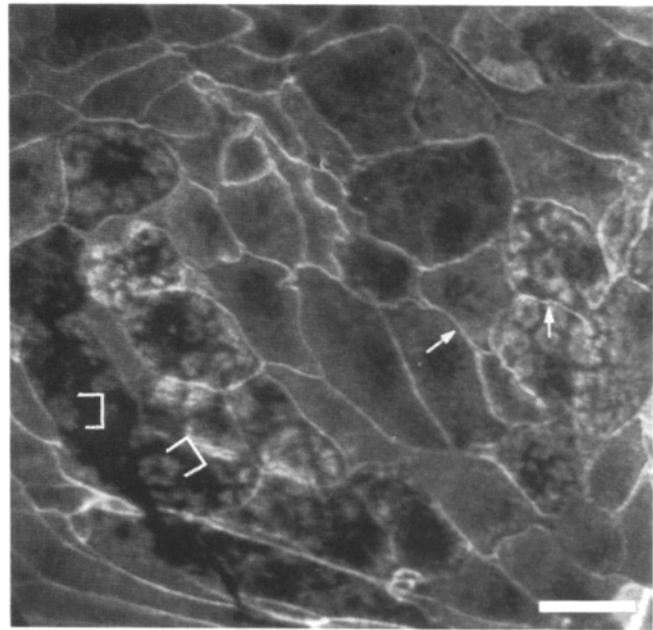


Figure 6. Localization of myosin-VI and actin in differentiated LLC-PK₁ cells. Coverslip-grown LLC-PK₁ cells 13 d after confluence were prepared for confocal immunofluorescence microscopy as described in Fig. 5. An optical section through the apical domain of the monolayer at the level of the tight junctions is shown. Microvilli appear as clustered spikes in the actin-stained panel (*right panel, brackets*). Junctions are indicated by small arrows. The myosin-VI (*left panel*) is not located to these actin-rich structures. Bar, 25 μ m.

and *b*, *solid arrows*), nor was it associated with actin cables (Fig. 5, *c* and *d*, *open arrows*) or stress fibers (Fig. 5, *e* and *f*, *arrowheads*) in subconfluent LLC-PK₁ cells. Rather, myosin-VI appeared as a diffuse, punctate stain within the cytoplasm of LLC-PK₁ cells. A similar staining pattern was also observed in differentiated CaCo-2_{BBC} cells (a human intestinal epithelial line; Peterson and Mooseker, 1992) and in the nonpolarized HeLa and NRK cell lines (not shown).

Possibly the lack of association of myosin-VI with the actin-cytoskeleton resulted from the undifferentiated state of the LLC-PK₁ cell line. Approximately 2 wk after the initiation of cell-cell contact, LLC-PK₁ (CLA) cells differentiate and develop an apical brush border. Immunofluorescence experiments using these differentiated monolayers indicated that this actin-rich domain also contained little myosin-VI staining (Fig. 6). Confocal imaging through the apical domain of an LLC-PK₁ monolayer showed clearly that the microvilli lacked myosin-VI staining (Fig. 6, *brackets*), although myosin-VI could be detected in the cytoplasm just below these structures (for example, cells marked with arrows in Fig. 6).

The cytoplasmic location of myosin-VI was also reflected in its subcellular fractionation profile. LLC-PK₁ cells were homogenized and fractionated by differential velocity sedimentation (Fig. 7 A). Visual inspection indicated that fractionation of LLC-PK₁ cells in this fashion sedimented apical brush borders and isolated nuclei into the low speed pellet, sheared microvilli, mitochondria, and other large membranous organelles into the high speed pellet, and small vesicles and organelles into the ultra speed pellet. Soluble components were recovered within the ultra speed superna-

tant. Myosin-II, which associates with the actin-rich subapical portion of the brush border and stress fibers within this cell line, sedimented into both the low and high speed pellets. Myosin-VI, however, in agreement with its cytoplasmic location, fractionated primarily into the ultra speed supernatant.

Myosins characteristically associate with microfilaments in an ATP-sensitive fashion. This was reflected in the fractionation profile of myosin-II. When LLC-PK₁ cells were homogenized in buffers containing 10 mM ATP, myosin-II was released from sedimenting structures and was now found primarily within the ultra speed supernatant. The fractionation profile of myosin-VI, however, was not significantly altered by the inclusion of ATP in the homogenization buffer. In both cases, myosin-VI was found primarily within the ultra speed supernatant. By comparison with myosin-VI tail fusion protein standards on immunoblots, we determined that myosin-VI constituted $\sim 0.8\%$ of total protein in LLC-PK₁ cells (data not shown).

Myosin-VI Binds F-Actin in an ATP-sensitive Fashion

Since porcine myosin-VI exhibited a high degree of identity to other myosins at the amino acid level, we felt it was important to assess whether this protein exhibited any myosin-like properties. First, we sought to address whether porcine myosin-VI could bind actin. Ultra speed supernatants from fractionated LLC-PK₁ cells were mixed with F-actin in the presence or absence of 10 mM ATP, and the sedimentation of extract components assayed by immunoblot. As shown in Fig. 8, a portion of both the soluble myosin-VI and -II was

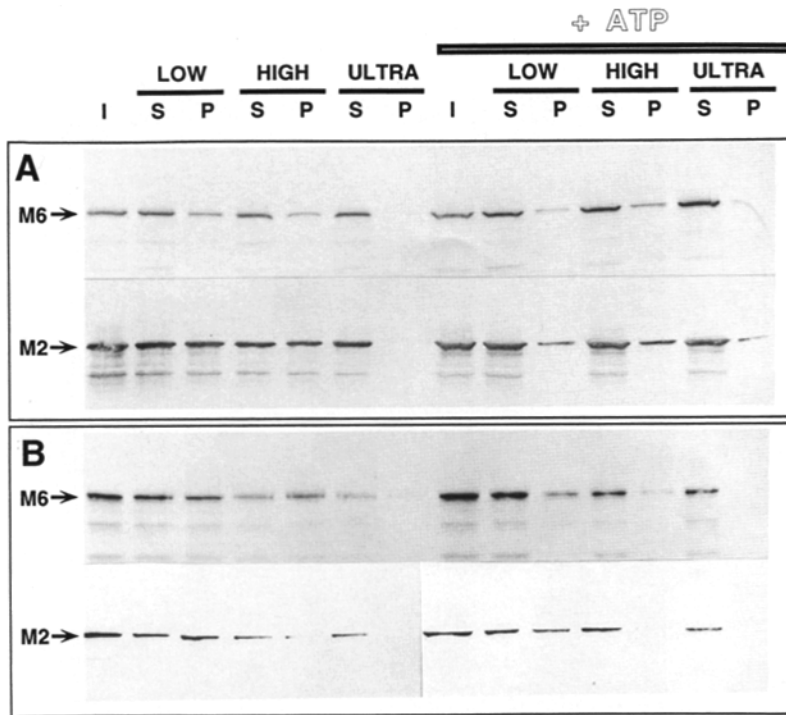


Figure 7. Myosin-VI exhibits a different subcellular fractionation pattern between LLC-PK₁ cells and kidney. (A) LLC-PK₁ cells or (B) whole mouse kidneys were homogenized and fractionated by differential sedimentation. The initial homogenization was done in the presence or absence of 10 mM ATP (right side of panel, +ATP). Samples from this fractionation included the whole-cell homogenate (I, input) and low, high, and ultra speed supernatants (S) and pellets (P). Fractions were brought up to equal volumes, and samples of each fraction were separated on a 5–16% polyacrylamide gel and blotted to nitrocellulose. Blots were probed with either affinity-purified rabbit anti-myosin-VI antibodies (M6) or rabbit anti-human platelet myosin-II antibodies (M2). Arrows indicate the appropriately sized bands. Myosin-II is included as an example of ATP-sensitive sedimentation. Myosin-II sediments into low and high speed pellet fractions in the absence of ATP, but in the presence of ATP, this association with the actin cytoskeleton is released, and the myosin-II immunogens are found within the ultra speed supernatant fractions. Myosin-VI exhibits this ATP-sensitive sedimentation profile only in kidney fractionations. Within LLC-PK₁ cells, myosin-VI is essentially soluble and fractionates into the ultra speed supernatant in the presence or absence of ATP.

capable of binding F-actin in this assay. In both cases, the actin-binding activity of the myosin was sensitive to ATP. Clearly, even though myosin-VI does not associate with actin-containing microfilaments within the cell line, this protein is capable of binding F-actin *in vitro*.

The association of myosin-VI with actin by cosedimentation could be seen on Coomassie-stained gels as well. In particular, a single 145-kD polypeptide, which comigrated with myosin-VI on immunoblots, was observed to bind F-actin in an ATP-sensitive fashion (Fig. 8, *arrowhead*).

Myosin-VI Is Associated with a Calmodulin Light Chain

The calcium-binding protein calmodulin is known to function as a light chain for many unconventional myosins, including brush border myosin-I, chick brain myosin-V, and *Drosophila* NinaC, a class III myosin (Espreafico et al., 1992; Howe and Mooseker, 1983; Porter et al., 1993). Given the presence of a single IQ motif in the neck domain of myosin-VI, we investigated whether porcine myosin-VI, like these other unconventional myosins, was associated with calmodulin.

Myosin-VI was immunoprecipitated, and its light chain composition was compared to purified chick brain myosin-V (p190) and chicken brush border myosin-II (Fig. 9). Eukaryotic nonmuscle myosin-II heavy chain is associated with a regulatory and an essential light chain. Both light chains are distinct from calmodulin, but are members of the calmodulin superfamily (reviewed in Korn and Hammer, 1988; Fig. 9 A). When samples of myosin-VI, -V, and -II were immunoblotted with an anti-calmodulin monoclonal antibody (Sacks et al., 1991), both the myosin-V- and myosin-VI-associated light chains were reacted with antibody (Fig. 9 B). The

myosin-II-associated light chains were not recognized by this calmodulin-specific probe. The myosin-VI light chain also exhibited the Ca⁺⁺-dependent mobility shift characteristic of calmodulin. Calmodulin has been shown to migrate at 21 kD in the absence of Ca⁺⁺ and 16 kD in the presence of Ca⁺⁺ (Burgess et al., 1980). A similar shift in mobility was observed when immunoprecipitated myosin-VI was incubated in the presence of 1 mM Ca⁺⁺ or 1 mM EGTA before electrophoresis (Fig. 9 B). This result confirmed that myosin-VI was associated with a calmodulin light chain.

Myosin-VI Is Associated with the Apical Domain of Proximal Tubule Cells in Adult Mouse Kidney

We also analyzed the location of myosin-VI within the kidney to determine whether the location of this myosin within the LLC-PK₁ cell line was reminiscent of its location within the proximal tubule. Surprisingly, immunofluorescence labeling of frozen thin sections of adult mouse kidney with the affinity-purified anti-myosin-VI antibodies identified specifically the actin-rich apical domain of proximal tubule cells (Fig. 10). In double-labeling experiments, fluorescein-labeled phalloidin bound to both the apical brush border and basal domains of the proximal tubule cells (*p*). The myosin-VI probe, however, specifically recognized only the apical domain. Basal actin-containing domains were not recognized.

Myosin-VI appeared to be specific for the proximal tubule brush border. Distal tubules (*d*), which have much sparser microvilli and, therefore, a less bright apical stain with phalloidin, were not recognized by the myosin-VI-specific antibody. The myosin-VI antibodies did not bind to other actin-rich domains of the kidney, such as the glomerulus (Fig. 10 *g*, lower panels) or the capillaries (*c*). In particular, the

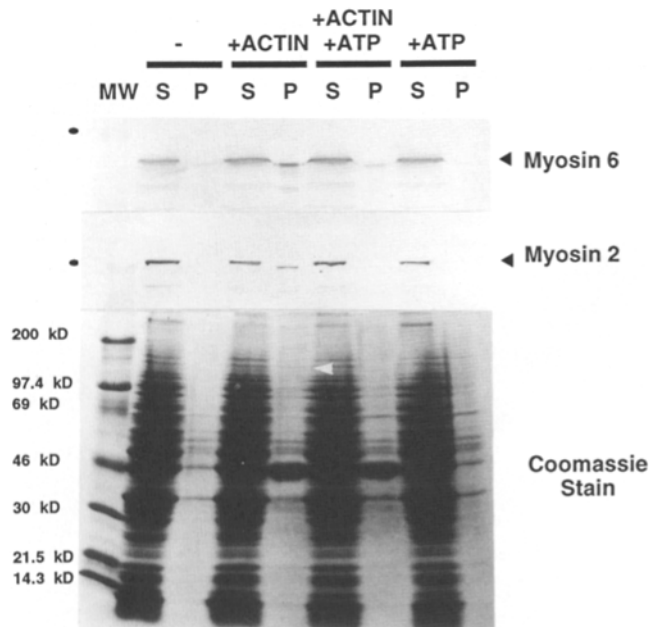


Figure 8. Myosin-VI binds F-actin in an ATP-sensitive fashion in cosedimentation assays. The ultra speed supernatant from fractionated LLC-PK₁ cells (Fig. 7) was incubated with F-actin, 10 mM ATP, or both F-actin and ATP and spun at 100,000 *g*. The resulting supernatants (*S*) and pellets (*P*) were resolved on a 5–16% polyacrylamide gel and were either stained with Coomassie blue, or immunoblotted and probed with affinity-purified rabbit anti-myosin-VI antibodies or rabbit anti-human platelet myosin-II antibodies. A portion of both myosin-VI and myosin-II are observed to cosediment with F-actin in the absence of ATP, but not in its presence. The sedimenting myosin-VI can also be visualized on the Coomassie-stained gel, and it is marked with an arrowhead. This 145-kD band is observed within pellet fractions only in the presence of F-actin and the absence of ATP, and it precisely comigrates with myosin-VI on immunoblots. Bullets indicate the 200-kD molecular weight marker (*MW*) on the immunoblots. Coomassie-stained molecular weight markers are delineated on the left in kilodaltons.

glomerulus is rich in actin-containing junctional complexes, but these structures were not recognized by the myosin-VI antibodies. Nonimmune IgG and secondary antibody alone controls did not recognize any of the kidney components (data not shown). In more collapsed proximal tubules, such as the structure marked with an asterisk in Fig. 10, it was evident that the myosin-VI stain was enriched at the base of the microvilli. These results suggest that this protein may be enriched within the vesicle-rich subapical cytoplasm of the proximal tubule cells.

This difference in location between LLC-PK₁ cells and kidney was also reflected in the sedimentation profile of myosin-VI. Homogenized pig (not shown) and mouse kidneys were fractionated by sedimentation as described previously for the LLC-PK₁ cell line (Fig. 7 *B*). Unlike the myosin-VI within the LLC-PK₁ cell line, the majority of the protein was found within the low and high speed pellet fractions, with only a small portion of the myosin within the ultra speed supernatant. As expected for an actin-associated myosin, this sedimentable myosin-VI was released upon homogenization in the presence of 10 mM ATP. Myosin-II exhibited a similar ATP-dissociable sedimentation. These results sug-

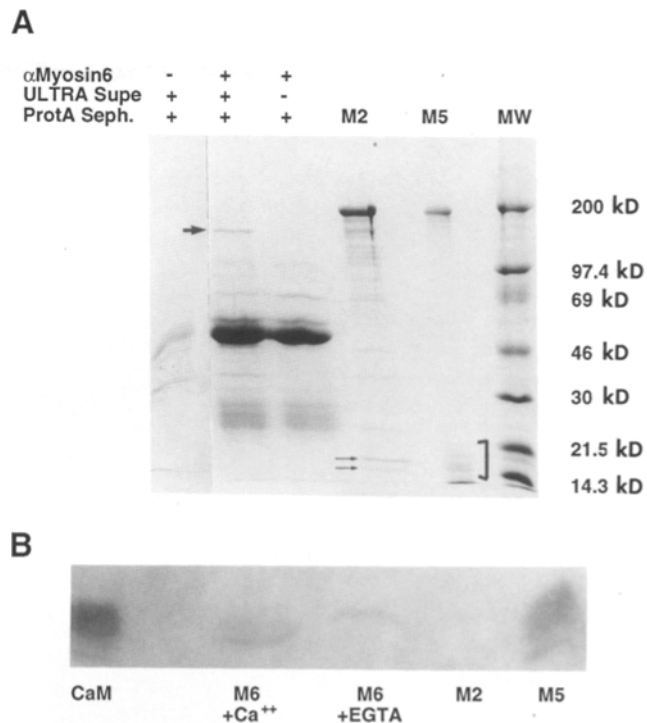


Figure 9. Myosin-VI is associated with a calmodulin light chain. (*A*) Coomassie-stained polyacrylamide gel of protein samples used for anti-calmodulin protein blots. Myosin-VI was immunoprecipitated from the ultra speed supernatant of fractionated LLC-PK₁ cells using 10 μ g affinity-purified rabbit anti-myosin-VI antibodies and 3 mg protein A-Sepharose CL4 beads. Negative controls included identical immunoprecipitations lacking either the primary antibody or the ultra speed supernatant. A single 145-kD polypeptide was specifically immunoprecipitated from these extracts, and it is marked with an arrow. 3 μ g purified chicken brush border myosin-II (*M2*) and 1 μ g purified chick brain myosin-V (*M5*) are shown for comparison. The essential and regulatory light chains associated with myosin-II are pointed out with small arrows. The myosin-V-associated calmodulin light chains are bracketed. Because the myosin-V sample was not prepared in the presence of excess Ca⁺⁺ or EGTA, the calmodulin light chains run as a set of four bands reflecting the different Ca⁺⁺-bound states of this polypeptide (Burgess et al., 1980). Purified calmodulin also exhibits this pattern (see *B*). Molecular weight markers (*MW*) are shown on the right in kilodaltons. (*B*) Immunoblot probing for calmodulin associated with immunoprecipitated myosin-VI and purified myosins-II and -V. Samples identical to those shown in *A* were blotted to PVDF membrane, and probed with a monoclonal antibody to calmodulin. 1 μ g purified bovine brain calmodulin was included as a positive control (*CaM*). 3 μ g each of purified chicken brush myosin-II and purified chick brain myosin-V were used in this experiment. The anti-calmodulin antibodies specifically recognized purified calmodulin and the calmodulin light chains associated with myosins-V and -VI. The regulatory and essential light chains associated with myosin-II were not recognized by this calmodulin-specific probe. After immunoprecipitation, the myosin-VI was boiled in sample buffer containing 10 mM Ca⁺⁺ (*M6* + Ca⁺⁺) or 10 mM EGTA (*M6* + EGTA) before loading on the gel. The presence or absence of Ca⁺⁺ did not affect the mobility of myosin-VI (data not shown), but it affected the mobility of the calmodulin light chains associated with myosin-VI. This shift in mobility from 16 to 20 kD in the absence of Ca⁺⁺ is characteristic of calmodulin, and it is not seen with other calmodulin-like proteins (Burgess et al., 1980).

Myosin 6

Actin

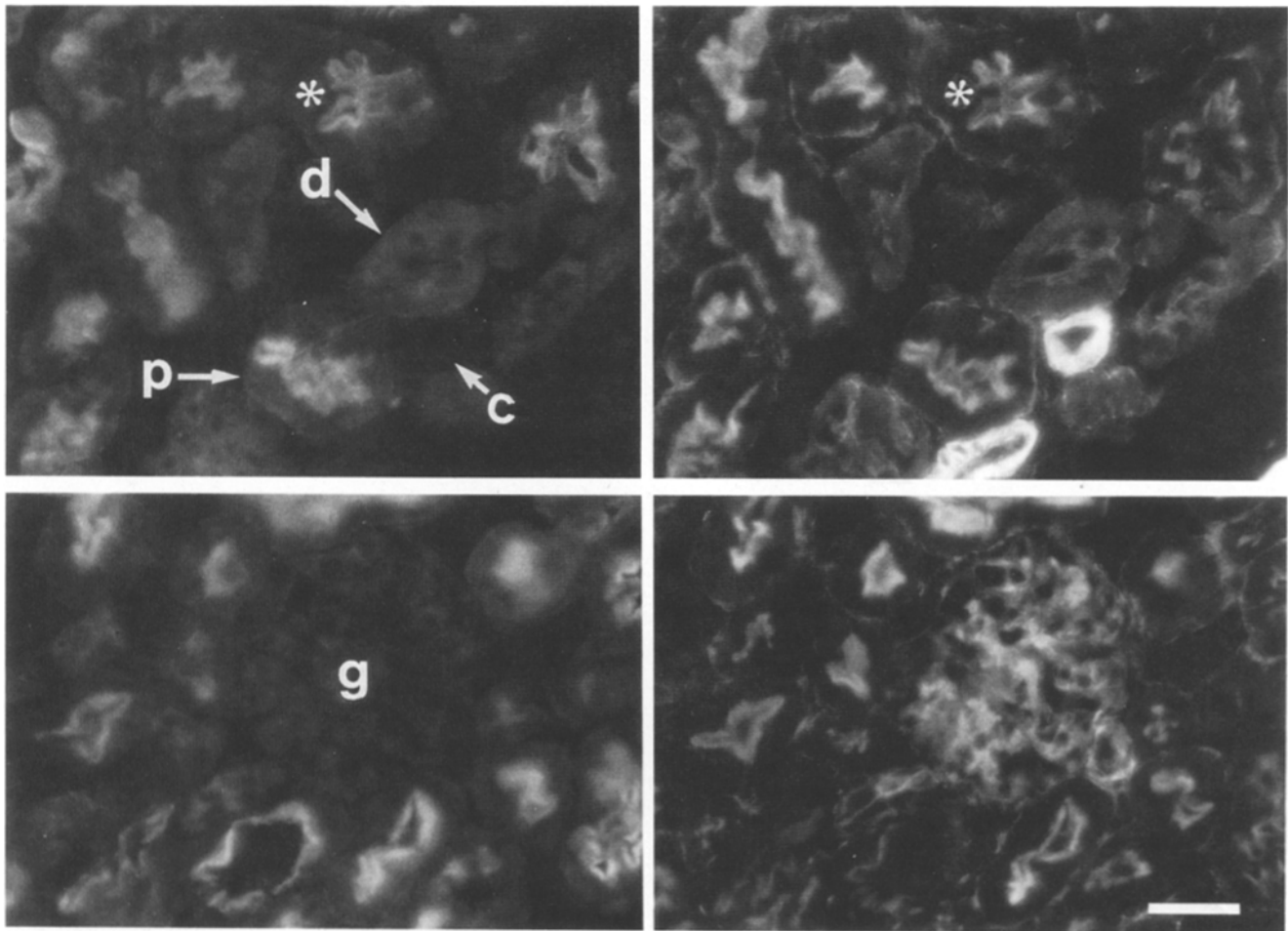


Figure 10. Indirect immunofluorescence localization of myosin-VI and actin within the kidney. Frozen thin sections of formaldehyde-fixed adult mouse kidney were incubated with 10 $\mu\text{g/ml}$ affinity-purified rabbit anti-myosin-VI antibodies and were also stained with fluorescein-labeled phalloidin to visualize F-actin. Sections showing proximal (*p*) and distal (*d*) tubules as well as the glomerulus (*g*) and the capillaries (*c*) of the kidney are shown. F-actin is present within all these kidney structures. Myosin-VI, however, was specifically located only to the actin-rich apical brush border domain of the proximal tubules. The myosin was not enriched in distal tubules, glomeruli, or capillaries of the kidney. Bar, 25 μm .

gest that myosin-VI is associated with the apical actin cytoskeleton within the proximal tubule cells of the adult kidney.

Discussion

We have cloned and characterized a new unconventional myosin, porcine myosin-VI, a homologue of *Drosophila* 95F MHC. This is the first myosin of this class isolated from a mammalian source. Myosin-VI, like other unconventional myosins, is associated with a calmodulin light chain, and it exhibits ATP-sensitive actin-binding activity. In cultured cells, myosin-VI is not associated with major F-actin-containing structures, but in the kidney, this motor localizes to the apical brush border domain of proximal tubule cells. The association of myosin-VI with sedimentable compartments within the kidney is ATP sensitive, suggesting that myosin-VI is associated with the actin cytoskeleton.

To date, in addition to myosin-II, three unconventional myosins have been identified within the kidney. A 105-kD calmodulin-binding protein was partially purified from rat kidney (Coluccio, 1991). This polypeptide bound F-actin in an ATP-sensitive fashion, and it exhibited ATPase activity in crude extracts, suggesting that it was a myosin. Antibodies directed against a 116-kD myosin-I purified from bovine adrenal glands, and identified by PCR from mouse brain recognize a related species in kidney (Sherr et al., 1993; Wagner et al., 1992; Reizes et al., 1994). Similarly, a 128-kD myosin-I, myr1b, identified in rat brain, is also expressed in this tissue as judged by Northern analysis and immunoblot (Ruppert et al., 1993). As judged by their sizes and differences in sequence, these myosins-I are all clearly distinct from the myosin-VI described in this work. LLC-PK₁ cells and kidney extracts both contain at least three 110–130-kD myosin-I immunogens when probed by immunoblot with antibodies that recognize epitopes shared by myosin-I head do-

mains (Fig. 3; unpublished data). Our PCR screen for myosins expressed in differentiating LLC-PK₁ cells identified nine different myosins, but only one myosin-I-like clone (Bement et al., 1994a). This clone is distinct from myr1b and the adrenal myosin-I at the amino acid level. It is likely that upon further study, even more myosin species that are expressed within this actin-rich tissue will be identified.

Myosin-VI and Assembly of the Brush Border

Myosin-VI does not appear to be required for brush border assembly in the proximal tubule cell. LLC-PK₁ cells express what appears to be a fully differentiated brush border as judged by the presence of brush border-specific markers such as villin, fimbrin, and myosin-II in the apical domain (Hasson, T., and M. S. Mooseker, unpublished observations; Temm-Grove et al., 1992). Also, LLC-PK₁ cells, upon differentiation, undergo many of the activities associated with proximal tubule kidney cells, including vectorial transport of D-glucose (Misfeldt and Sanders, 1981; Rabito and Ausiello, 1980), Na⁺-H⁺ exchange (Igarashi et al., 1991), and targeting of membrane proteins to apical and basal-lateral domains (Gottardi and Caplan, 1993). Inspection of these cells at the electron microscopic level, however, indicates that the brush border expressed by the cell line is less lush than that seen within the kidney (Pfaller et al., 1990; our observations). In addition, LLC-PK₁ cells grown on glass coverslips are much less columnar than the proximal tubule cells. The cell line differentiates to a size of ~4 μm tall, compared to the 20-μm cells observed within the kidney. Possibly, the soluble nature of myosin-VI within LLC-PK₁ cells was caused in part by these differences in the membrane cytoskeleton.

Since myosin-VI is tightly associated with the brush border in adult kidney proximal tubule cells, it is possible that LLC-PK₁ cells have not fully differentiated and that myosin-VI associates with the brush border late in this differentiation process. This theory correlates with our recent studies on the assembly of myosin-VI into the apical cytoskeleton of the proximal tubule during kidney differentiation in rats. We have observed that myosin-VI specifically locates to the apical domain only during the final stages of proximal tubule development, when brush border assembly has been completed (Hasson, T., and D. Biemesderfer, unpublished observations). Clearly, the assembly of myosin-VI into the actin cytoskeleton in vivo requires a developmental signal; we suggest that this signal is not provided during the differentiation of LLC-PK₁ cells induced by cell-cell contact.

This is not the first example of the late incorporation of a myosin into the brush border. In studies of the differentiation of enterocytes along the crypt/villus axis in chicken, it was observed that the microvillar component brush border myosin-I also undergoes late association with the apical domain (Heintzelman and Mooseker, 1990). This unconventional myosin did not assume an apical location until the emergence of the enterocyte from the crypt into the villus, a stage when brush border assembly was essentially complete. By comparison, the microvillar components actin, villin, and fimbrin were all polarized in their location at the onset of enterocyte differentiation, and they were associated with the apical domain along the entire crypt/villus axis (Heintzelman and Mooseker, 1990). It is not known what

Porcine M6	SLTTRVMLTTAGGAKGTVIKPLLKVEQANNARDALAK	426
Dro 95F	ALVSRVMQSKGGGFKGTVMVPLKIYEASNARDALAK	425
Aca M1A	AGTTYALNLNKMQAIGSRDALAK	N.D.
Aca M1B	LLFRVLNTGGAGAKMSTYNVFPQNVQEAASARDALAK	335
Aca M1C	LLYRTITTGEQGRSSVYSCPDPLGAIYSRDLASK	331
Dict M1A	SSLVSRQISTGGGARISTYSVPQTVQAMYARDAFAK	354
Dict M1B	FRVINTGGAGGAGNRSTYNVFPQNVQANGTRDALAR	352
Dict M1D	CFRTISTGTQGRSARVSTYACFQNSEGAYYSRDLAK	352
Dict M1E	IALCYRSISTGVGKRCSVISVFNDCNQAAYSRLDALAK	356

Figure 11. Comparison of head sequences between myosins-VI and amoeboid myosins-I at the site of phosphorylation by amoeboid myosin heavy chain kinase. This analysis expands the alignment of amoeboid myosin head domain phosphorylation sites first described in Brzeska et al. (1989). In this study, the sequence surrounding the phosphorylated amino acid of *Acanthamoebae* myosins-IA, -IB, and -IC was identified by peptide sequencing. In our alignment, we have added the amino acid sequences of the more recently cloned *Dictyostelium* myosins-I (Jung et al., 1989, 1993; Titus et al., 1989; Urrutia et al., 1993) and the myosins-VI, porcine myosin-VI, and *Drosophila* 95F MHC (Kellerman and Miller, 1992). All these myosins have a conserved serine or threonine residue at the presumed phosphorylation site (boldface). Note the high degree of identity between the region phosphorylated in *Dictyostelium* myosin-IE and the corresponding region of the myosins-VI. ND, Not determined to date.

signal is required to initiate the assembly of this myosin-I into the brush border.

Myosin-VI in Other Brush Border-containing Cells

Myosin-VI is also expressed in other brush border-containing cells. Protein blots indicate that this protein is expressed within intestinal epithelial cells and within the epididymus of the testes (Fig. 4; Heintzelman, M., unpublished observations). We have more carefully characterized the intestinal myosin-VI and have found that, as seen in kidney, myosin-VI is specifically associated in an ATP-sensitive fashion with the brush border domain of chicken intestinal epithelial cells (Heintzelman et al., 1994). Intestinal myosin-VI was predominantly localized to the terminal web domain, while lower levels of expression were also seen within the microvilli. Surprisingly, ~30% of the intestinal myosin was found to be soluble within the epithelia. This is in sharp contrast with the myosin-VI in kidney, which is much more tightly associated with the actin-cytoskeleton.

To characterize the assembly of myosin-VI into the enterocyte brush border, the association of myosin-VI with the apical cytoskeleton of intestinal epithelia was observed during enterocyte differentiation along the villus/crypt axis. In results similar to those described above for brush border myosin-I, myosin-VI was observed to locate to the apical domain of the chicken enterocyte only after the cells emerged from the crypt into the villus (Heintzelman et al., 1994). Within crypt cells, myosin-VI had a diffuse cytoplasmic stain not unlike that observed within the LLC-PK₁ cell line. Clearly, the association of myosin-VI with the actin cytoskel-

eton is highly regulated in both kidney and intestine. This forces us to question what signal is required for the proper location of myosin-VI to the apical domain.

Regulation of Myosin-VI: Heavy Chain Phosphorylation?

Among known myosins, only amoeboid myosins-I have been shown to be regulated by heavy chain phosphorylation of their head domain (reviewed in Tan et al., 1992). As described elegantly by Brzeska and colleagues (1989), a single serine or threonine residue within the head domain of *Acanthamoeba* myosins-I is specifically phosphorylated by amoeboid myosin heavy chain kinase (Fig. 11). Interestingly, as pointed out in Bement et al. (1994b), both porcine and *Drosophila* myosins-VI also have a threonine at this conserved position. In the case of *Dictyostelium* myosins-IB and -ID, as well as all the *Acanthamoeba* myosins-I, this phosphorylation is required for maximal actin-activated ATPase activity and motility (reviewed in Tan et al., 1992; Lee and Côté, 1993). Crystallographic analysis of myosin S1 bound to F-actin indicates that this region of the myosin head makes direct contacts with actin (Rayment et al., 1993a; Schröder et al., 1993). Given the evolutionary conservation of charge at this actin-myosin contact site, the presence of a threonine residue within this domain of the myosin-VI head raises the possibility that myosins-VI will also require heavy chain phosphorylation at this location for maximal activity. As evidence for the importance of this domain, humans with a single arginine to glutamine missense mutation within this domain in β -cardiac myosin-II exhibit hypertrophic cardiomyopathy (Geisterfer-Lowrance et al., 1990). Expression or induction of a kinase may be the signal required for assembly of myosin-VI into the apical cytoskeleton. This need for activation by heavy chain phosphorylation may well explain the lack of complete binding by myosin-VI to pure F-actin in co-sedimentation assays (Fig. 8).

Regulation of Myosin-VI: Light Chains?

Myosin-VI assembly with the apical domain, as well as its intrinsic actin-activated ATPase activity, may also be regulated by its associated light chain(s). Foremost, myosins require the presence of their proper regulatory and essential light chains to maintain physiological actin-activated ATPase activity and motility (Collins et al., 1990; Trybus et al., 1994, and references therein; Wolenski et al., 1993). The conserved 50-aa shift in the location of the myosin-VI calmodulin binding (IQ) motif may indicate that myosins-VI bind a unique light chain within this region. Such a novel light chain would not have been detected using the calmodulin-specific antibodies used in this study, and could serve to regulate this motor.

A novel regulatory light chain could also be further modified. The phosphorylation of regulatory light chains associated with conventional myosins leads to a specific activation of the actin-activated ATPase activity of these motors, and it induces the assembly of myosin-II filaments (reviewed in Korn and Hammer, 1988; Warrick and Spudich, 1987). Myosins-VI harbor a coiled-coil domain and, therefore, may be found as a dimer or form filaments within the cell. A novel light chain could serve to regulate this change in assembly.

The calmodulin light chain that we have shown to be associated with myosin-VI may also serve to regulate the motor. The binding of calmodulin light chains to Ca^{++} has been shown, in vitro, to regulate the actin-activated ATPase activity of a number of unconventional myosins, such as brush border myosin-I and chick brain myosin-V (Wolenski et al., 1993; Collins et al., 1990; Cheney et al., 1993a). Myosin-VI was immunoprecipitated from homogenates in the presence of EGTA; it is unknown whether the presence of Ca^{++} would release the calmodulin light chain from the myosin-VI heavy chain, as has been seen for other unconventional myosins (Cheney et al., 1993a; Wolenski et al., 1993).

Regulation of Myosin-VI: Association with a Cellular Cargo?

The assembly of myosin-VI with the apical cytoskeleton may be affected by the binding of a cellular component or "cargo." The tail domain of the myosin-VI class is unique and highly conserved between pig and fly. Possibly through this novel tail, myosin-VI binds to a conserved docking protein expressed only in fully differentiated cells. 95F MHC has been found to be tightly associated with cytoplasmic particles within the developing fly embryo (Mermall et al., 1994). These particles have been observed to move within the embryo in an ATP- and actin-dependent fashion. As these particles move into the pseudo-cleavage furrow during mitosis, it is possible these structures represent membrane organelles. Porcine myosin-VI is not tightly membrane associated, however, as judged by its ready extraction from cell homogenates with ATP. Mammalian myosins-I, which are known to be membrane associated, require high salt or detergent in addition to ATP to be released from the membrane cytoskeleton (Fath and Burgess, 1993; Howe and Mooseker, 1983). These myosins have also been shown to bind acidic phospholipids in vitro (Hayden et al., 1990). We have been unable to observe any specific binding of porcine myosin-VI to acidic phospholipids in vitro, using either immunoprecipitated myosin-VI or myosin-VI tail fusion proteins (unpublished observations). It is conceivable that the tail portion of myosin-VI affords an interaction with a membrane-associated protein. Such a protein-protein interaction would link this membrane compartment with the actin cytoskeleton. This protein-protein link could have been disrupted during our fractionation procedures. Analysis of myosin-VI location at the electron microscope level will be critical to determine whether this myosin is indeed associated with a membrane compartment within the proximal tubule cell.

What Is the Function of Myosin-VI?

Myosin-VI is a ubiquitous protein, and it is expressed in many tissues that do not contain a brush border. For example, myosin-VI is expressed in high levels in brain. What function might a myosin serve in such varied tissue types? The expression of myosin-VI specifically within the apical brush border of the proximal tubule cells may give us a clue. Unlike distal tubules within the kidney, the proximal tubule serves as a major site of endocytosis (reviewed in Christensen, 1982). As a result, the apical domain of this cell type is filled with clathrin-coated vesicles and other membranes. This endocytosis is not seen in the LLC-PK₁ cell line, and

it reflects a major difference between this proximal tubule model and functioning kidney. Perhaps myosin-VI serves a role in these membrane movements.

The extensive membrane recycling that occurs within the apical domain of the proximal tubule requires an active membrane internalization, retrieval, and reutilization apparatus. The polarized actin filaments present in the brush border would provide an excellent roadway for the return of membranes to the apical surface. Myosin-VI, if it does indeed associate with membranes, may serve to transiently anchor these membranes onto the actin cytoskeleton and return them to the apical membrane. Such an activity would be required within all tissues, including those lacking a brush border domain. The availability of myosin-VI-specific probes should allow us to more fully characterize the role this motor plays in the movement of components in both polarized and nonpolarized cells.

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References

- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York. pp. 4.9.1-4.9.5.
- Bement, W. M., P. Forscher, and M. S. Mooseker. 1993. A novel cytoskeletal structure involved in purse string wound closure and cell polarity maintenance. *J. Cell Biol.* 121:565-578.
- Bement, W. M., T. Hasson, J. A. Wirth, R. E. Cheney, and M. S. Mooseker. 1994a. Identification and overlapping expression of multiple unconventional myosin genes in vertebrate cell types. *Proc. Natl. Acad. Sci. USA.* 91:6549-6553.
- Bement, W. M., J. A. Wirth, and M. S. Mooseker. 1994b. Cloning and mRNA expression of human unconventional myosin-IC: a homologue amoeboid myosin-I with a single IQ motif and an SH3 domain. *J. Mol. Biol.* In press.
- Brzeska, H., T. J. Lynch, B. Martin, and E. D. Korn. 1989. The localization and sequence of the phosphorylation sites of *Acanthamoeba* myosins I. *J. Biol. Chem.* 264:19340-19348.
- Burgess, W. H., D. K. Jemio, and R. H. Kretsinger. 1980. Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate. *Biochim. Biophys. Acta.* 623:257-270.
- Carboni, J. M., K. A. Conzelman, R. A. Adams, D. A. Kaiser, T. D. Pollard, and M. S. Mooseker. 1988. Structural and immunological characterization of the myosin-like 100-kD subunit of the intestinal microvillar 110K calmodulin complex: evidence for discrete myosin head and calmodulin-binding domains. *J. Cell Biol.* 107:1749-1757.
- Cheney, R. E., and M. S. Mooseker. 1992. Unconventional myosins. *Curr. Opin. Cell Biol.* 4:27-35.
- Cheney, R. E., M. K. O'Shea, J. E. Heuser, M. V. Coelho, J. S. Wolenski, E. M. Espreafico, P. Forscher, R. E. Larson, and M. S. Mooseker. 1993a. Brain myosin-V is a two-headed unconventional myosin with motor activity. *Cell.* 75:13-23.
- Cheney, R. E., M. A. Riley, and M. S. Mooseker. 1993b. Phylogenetic analysis of the myosin superfamily. *Cell Motil. Cytoskel.* 24:215-223.
- Christensen, E. I. 1982. Rapid membrane recycling in renal proximal tubule cells. *Eur. J. Cell Biol.* 29:43-49.
- Collins, K., J. R. Sellers, and P. Matsudaira. 1990. Calmodulin dissociation regulates brush border myosin-I (110-kD calmodulin) mechanochemical activity in vitro. *J. Cell Biol.* 110:1137-1147.
- Coluccio, L. M. 1991. Identification of the microvillar 110-kDa calmodulin complex (myosin-I) in kidney. *Eur. J. Cell Biol.* 56:286-294.
- Devereaux, J., P. Haerberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387-395.
- Espreafico, E. M., R. E. Cheney, M. Matteoli, A. A. C. Nascimento, P. V. De Camilli, R. E. Larson, and M. S. Mooseker. 1992. Primary structure and localization of chicken brain myosin-V (p190), an unconventional myosin with calmodulin light chains. *J. Cell Biol.* 119:1541-1557.
- Fath, K. R., and W. H. Burgess. 1993. Golgi-derived vesicles from developing epithelial cells bind actin filaments and possess myosin-I as a cytoplasmically oriented peripheral membrane protein. *J. Cell Biol.* 120:117-127.
- Forscher, P., and S. J. Smith. 1988. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol.* 107:1505-1516.
- Geisterfer-Lowrance, A. A. T., S. Kass, G. Tanigawa, H.-P. Vosberg, W. McKenna, C. E. Seidman, and J. G. Seidman. 1990. A molecular basis for familial hypertrophic cardiomyopathy: A β cardiac myosin heavy chain gene missense mutation. *Cell.* 62:999-1006.
- Goodson, H. V., and J. A. Spudich. 1993. Molecular evolution of the myosin family: relationships derived from comparisons of amino acid sequences. *Proc. Natl. Acad. Sci. USA.* 90:659-663.
- Gottardi, C. J., and M. J. Caplan. 1993. An ion-transporting ATPase encodes multiple apical localization signals. *J. Cell Biol.* 121:283-293.
- Haggerty, J. G., N. Agarwal, R. F. Reilly, E. A. Adelberg, and C. W. Slayman. 1988. Pharmacological different Na/H antiporters on the apical and basolateral surfaces of cultured porcine kidney cells (LLC-PK₁). *Proc. Natl. Acad. Sci. USA.* 85:6797-6801.
- Halsall, D. J., and J. A. Hammer III. 1990. A second isoform of chicken brush border myosin-I contains a 29 residue inserted sequence that binds calmodulin. *FEBS (Fed. Eur. Biochem. Soc.)* 267:126-130.
- Handler, J. S. 1986. Studies of kidney cells in culture. *Kidney Int.* 30:208-215.
- Hayden, S. M., J. Wolenski, and M. S. Mooseker. 1990. Binding of brush border myosin-I to phospholipid vesicles. *J. Cell Biol.* 111:443-452.
- Heintzelman, M. B., T. Hasson, and M. S. Mooseker. 1994. Multiple unconventional myosin domains of the intestinal brush border cytoskeleton. *J. Cell Sci.* In press.
- Heintzelman, M. B., and M. S. Mooseker. 1990. Assembly of the brush border cytoskeleton: changes in the distribution of microvillar core proteins during enterocyte differentiation in adult chicken intestine. *Cell Motil. Cytoskel.* 15:12-22.
- Hoshimaru, M., and S. Nakanishi. 1987. Identification of a new type of mammalian myosin heavy chain by molecular cloning. *J. Biol. Chem.* 262:14625-14632.
- Howe, C. L., and M. S. Mooseker. 1983. Characterization of the 110-kD actin-, calmodulin-, and membrane-binding protein from microvilli of intestinal epithelial cells. *J. Cell Biol.* 97:974-985.
- Hull, R. N., W. R. Cherry, and G. W. Weaver. 1976. The origin and characteristics of a pig kidney cell strain, LLC-PK₁. *In Vitro.* 12:670-677.
- Igarashi, P., R. F. Reilly, F. Hildebrandt, D. Biemesderfer, N. A. Reboucas, C. W. Slayman, and P. S. Aronson. 1991. Molecular biology of renal Na⁺-H⁺ exchangers. *Kidney Int.* 40:584-S-89.
- Jung, G., C. L. Saxe III, A. R. Kimmel, and J. A. Hammer III. 1989. *Dicryostelium discoideum* contains a gene encoding a myosin-I heavy chain isoform. *Proc. Natl. Acad. Sci. USA.* 86:6186-6190.
- Jung, G., Y. Fukui, B. Martin, and J. A. Hammer III. 1993. Sequence, expression pattern, intracellular localization and targeted disruption of the *Dicryostelium* myosin-ID heavy chain isoform. *J. Biol. Chem.* 268:14981-14990.
- Kellerman, K. A., and K. G. Miller. 1992. An unconventional myosin heavy chain gene from *Drosophila melanogaster*. *J. Cell Biol.* 119:823-834.
- Korn, E. D., and J. A. Hammer III. 1988. Myosins of nonmuscle cells. *Annu. Rev. Biophys. Chem.* 17:23-45.
- Kozak, M. 1984. Compilation and analysis of sequences upstream from the translation start site in eukaryotic mRNAs. *Nucleic Acids Res.* 12:857-872.
- Lee, S.-F., and G. P. Côté. 1993. Isolation and characterization of three *Dicryostelium* myosin-I isozymes. *J. Biol. Chem.* 268:20923-20929.
- Lupas, A., M. VanDyke, and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science (Wash. DC)* 252:1162-1164.
- Maita, T., E. Yajima, S. Nagata, T. Miyayoshi, S. Nakayama, and G. Matsuda. 1991. The primary structure of skeletal muscle myosin heavy chain: IV. Sequence of the rod and the complete 1,938-residue sequence of the heavy chain. *J. Biochem.* 110:75-87.
- Mercer, J. A., P. K. Seperack, M. C. Strobel, N. G. Copeland, and N. A. Jenkins. 1991. Novel myosin heavy chain encoded by murine dilute coat

- colour locus. *Nature (Lond.)* 349:709-713.
- Mermall, V., J. G. McNally, and K. G. Miller. 1994. Transport of cytoplasmic particles catalysed by an unconventional myosin in living *Drosophila* embryos. *Nature (Lond.)* 369:560-562.
- Miller, K. G., C. M. Field, and B. M. Alberts. 1989. Actin-binding proteins from *Drosophila* embryos: a complex network of interacting proteins detected by F-actin affinity chromatography. *J. Cell Biol.* 109:2963-2975.
- Misfeldt, D. S., and M. J. Sanders. 1981. Transepithelial transport in cell culture: D-Glucose transport by a pig kidney cell line (LLC-PK₁). *J. Membr. Biol.* 59:13-18.
- Mooseker, M. S., T. D. Pollard, and K. Fujiwara. 1978. Characterization and localization of myosin in the brush border of intestinal epithelial cells. *J. Cell Biol.* 79:444-453.
- Mooseker, M. 1993. A multitude of myosins. *Curr. Biol.* 3:245-248.
- Peterson, M. D., and M. S. Mooseker. 1992. Characterization of the enterocyte-like brush border cytoskeleton of the C2_{BBC} clones of the human intestinal cell line, Caco-2. *J. Cell Sci.* 102:581-600.
- Pfaller, W., G. Gstraunthaler, and P. Loidl. 1990. Morphology of the differentiation and maturation of LLC-PK₁ epithelia. *J. Cell. Phys.* 142:247-254.
- Pollard, T. D., S. K. Doberstein, and H. G. Zot. 1991. Myosin-I. *Annu. Rev. Physiol.* 53:653-681.
- Porter, J. A., M. Yu, S. K. Doberstein, T. D. Pollard, and C. Montell. 1993. Dependence of calmodulin localization in the retina on the NINAC unconventional myosin. *Science (Wash. DC)* 262:1038-1042.
- Rabito, C. A., and D. A. Ausiello. 1980. Na⁺-dependent sugar transport in a cultured epithelial cell line from pig kidney. *J. Membr. Biol.* 54:31-38.
- Rayment, I., H. M. Holden, M. Whittaker, C. B. Yohn, M. Lorenz, K. C. Holmes, and R. A. Milligan. 1993a. Structure of the actin-myosin complex and its implications for muscle contraction. *Science (Wash. DC)* 261:5865.
- Rayment, I., W. R. Rypniewski, K. Schmidt-Bäse, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelman, G. Wesenberg, and H. M. Holden. 1993b. Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science (Wash. DC)* 261:50-58.
- Reizes, O., B. Barylko, C. Li, T. C. Südhof, and J. P. Albanesi. 1994. Domain structure of a mammalian myosin-1 β . *Proc. Natl. Acad. Sci. USA* 91:6439-6453.
- Rodman, J. S., D. Kerjaschki, E. Merisko, and M. G. Farquhar. 1984. Presence of an extensive clathrin coat on the apical plasmalemma of the rat kidney proximal tubule cell. *J. Cell Biol.* 98:1630-1636.
- Rodman, J. S., M. Mooseker, and M. G. Farquhar. 1986. Cytoskeletal proteins of the rat kidney proximal tubule brush border. *Eur. J. Cell Biol.* 42:319-327.
- Ruppert, C., R. Kroschewski, and M. Bähler. 1993. Identification, characterization, and cloning of myr1, a mammalian myosin-I. *J. Cell Biol.* 120:1393-1403.
- Sacks, D. B., S. E. Porter, J. H. Ladenson, and J. M. McDonald. 1991. Monoclonal antibody to calmodulin: development, characterization and comparison with polyclonal anti-calmodulin antibodies. *Anal. Biochem.* 194:369-377.
- Schröder, R. R., D. J. Manstein, W. Jahn, H. Holden, I. Rayment, K. C. Holmes, and J. A. Spudich. 1993. Three-dimensional atomic model of F-actin decorated with Dictyostelium myosin S1. *Nature (Lond.)* 364:171-174.
- Sherr, E. H., M. P. Joyce, and L. A. Greene. 1993. Mammalian myosin-1 α , 1 β , and 1 γ : new widely expressed genes of the myosin-I family. *J. Cell Biol.* 120:1405-1416.
- Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene (Amst.)* 67:31-40.
- Tan, J. L., S. Ravid, and J. A. Spudich. 1992. Control of nonmuscle myosins by phosphorylation. *Annu. Rev. Biochem.* 61:721-759.
- Temm-Grove, C., D. Helbing, C. Wiegand, B. Honer, and B. M. Jockusch. 1992. The upright position of brush border-type microvilli depends on myosin filaments. *J. Cell Sci.* 101:599-610.
- Titus, M. A., H. M. Warrick, and J. A. Spudich. 1989. Multiple actin-based motor genes in *Dictyostelium*. *Cell Regul.* 1:55-63.
- Trybus, K. M., G. S. Waller, and T. A. Chatman. 1994. Coupling of ATPase activity and motility in smooth muscle myosin is mediated by the regulatory light chain. *J. Cell Biol.* 124:963-969.
- Urrutia, R. A., G. Jung, and J. A. Hammer III. 1993. The *Dictyostelium* myosin-1E heavy chain gene encodes a truncated isoform that lacks sequences corresponding to the actin binding site in the tail. *Biochim. Biophys. Acta* 1173:225-229.
- Wagner, M. C., B. Barylko, and J. P. Albanesi. 1992. Tissue distribution and subcellular localization of mammalian myosin-I. *J. Cell Biol.* 119:163-170.
- Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay. 1982. Distantly related sequences in the α - and β -subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:945-951.
- Warrick, H. M., and J. A. Spudich. 1987. Myosin structure and function in cell motility. *Annu. Rev. Cell Biol.* 3:379-421.
- Wolenski, J. S., S. M. Hayden, P. Forscher, and M. S. Mooseker. 1993. Calcium-calmodulin and regulation of brush border myosin-I MgATPase and mechanochemistry. *J. Cell Biol.* 122:613-621.