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-PK_1 (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-PK_1 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-PK_1 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., 1993*b*; Goodson and Spudich, 1993; Bement et al., 1994*a*). 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.

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Using PCR technology, we undertook a characterization of the myosin isoforms expressed within the proximal tubule cells (Bement et al., 1994*a*). As a model for the proximal tubule, we used the porcine kidney cell line, LLC-PK_1, 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-PK_1 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., 1994*a*). These clones included two conventional myosins-II, consistent with the observation that myosin-II is expressed within the terminal web of the LLC-PK_1 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

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 NH_2-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-PK_1 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-PK_1 (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-PK_1 myosin clone (GenBank accession no. L29132; Bement et al., 1994*a*) was ^{32}P-labeled and used to screen a λZAP cDNA library prepared from LLC-PK_1 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 oligonucleotides 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-PK_1 λZAP 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 λZAP 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 XL1-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 μg/ml and sodium azide to 0.02%.

Northern Blots

Total RNA was isolated from LLC-PK_1 (clone 4) 4 d after confluence using the guanidinium lysis-CsCl gradient purification technique (Ausubel et al., 1989). 10 μg 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 ^{32}P-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 μg/ml aprotinin, 5 μg/ml pepstatin, 4.5 μg/ml chymostatin, and 5 μg/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., 1993*a*). 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 μg/ml as a primary antibody, monoclonal anti-calmodulin antibody (Upstate Biotechnology Inc., Lake Placid, NY) was used at a concentration of 0.5 μg/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-PK_1 cells were washed with PBS before scraping into buffer A (10 mM imidazole, pH 7.2, 75 mM KCl, 5 mM $MgCl_2$, 0.2 mM DTT, 1 mM K-EGTA, 0.2 mM PMSF, 5 μg/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 ± 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.

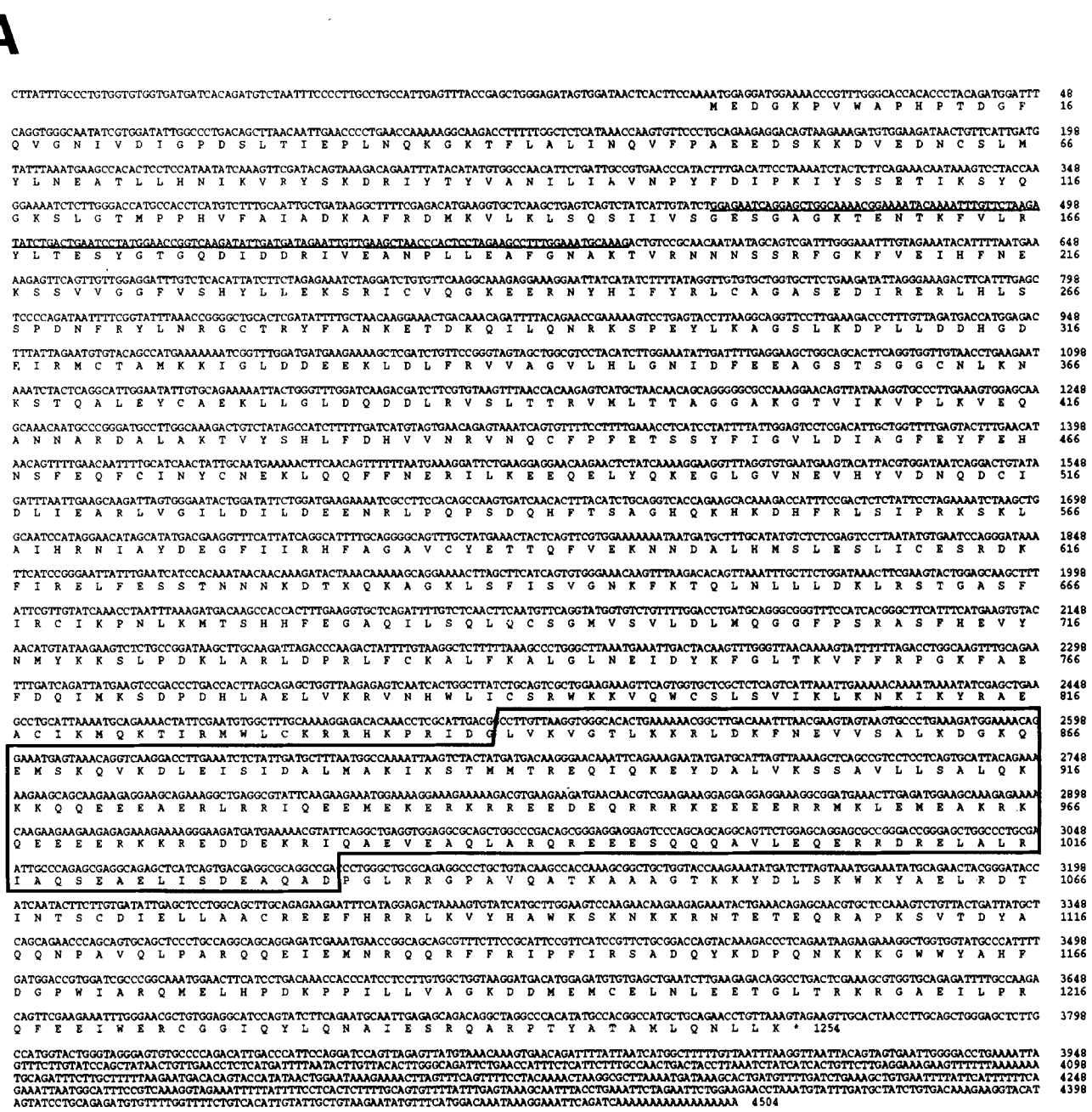

Figure 1. cDNA sequencing of porcine myosin-VI. (*A*) Nucleotide and deduced amino acid sequence of the porcine myosin-VI cDNA. Nucleotides are numbered relative to the start of the initiation codon. Below the sequence, the deduced amino acid sequence is shown starting from the initiator methionine. The TAG stop codon is indicated by an asterisk. A possible polyadenylylation 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., 1994*a*). 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/DDBJ under accession number Z35331. (*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.

A

B

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                1                                                                                                  100
Pig M-VI        .......... .......... .......... .MEDgkpVWe PhPtDGPqvG nIvDIGpDsL tIePLnqkGk tfLalinqVf paE......e DakkdVEDnC
 Dro 95f        .......... .......... .......... MLEDtqlVWV rdaaBGYIqG rItEIGakeF eVtPtdrkyp KrtchfDDI. hSs......c DQpqdhDDnC
Bov BBMI        .......... .......... .......... .......... .......... .......... .....Mtll EGaVgVEDLv
 Mus M-V        .......... .....mAase LytkfarVWI PdPeEvWksa ellkdyKpGd KVllLhleeG KdLeyrlDpk tgElphLrNP DilVgenDLt
Chk M-II        aspdaamaaf gaaapylrks ekerieAqnk pFDaksaVFV vhPkEsFVkG tI..qsKEGg KV.tvkteGG etLtvkEDqv fS.....mNP pkydkIEDMa
Consensus       ---------- ---------- ------A--- --ED---VWV P-P-EGF--G -I--IGK-G- KV-PL---GG K-L----D-- -SE-----NP DG-V-VEDLC

                101                                                                                ATP Binding Site 200
Pig M-VI        sLMYLnEatL LHNiKVRYsk Dr.IYTYVaN ILIAVNPYfd iPkIYssEtI KsYqGKSLGt MPPHVFAIAD KAFrDMkVlk lsQSIIVSGE SGAGKTEnTK
 Dro 95f        eLMlLnEatF LdNLKtRYyk DK.IYTYVaN ILIAVNPYre ikelYaPDtI KkYnGrSLGE LPPHVFAIAD KAirDMRVyk lsQSIIVSGE SGAGKTEsTK
Bov BBMI        LLepLeqesL irNLqlRY.e kKeIYTYIGN VLVsVNPYqq LP.IYdlSfV akYRdytFyE LkPHIYAlAn mAYqsLRdrd rdQcIlItGE SGAGKTEasK
 Mus M-V        aLsYLhEpav LHNLrVRFid sKLIYTYcGi VLVAINPYeq LP.IYgeDiI naYsGqnMGD MdPHIFAVAE eAYkqMarDe rnQSIIVSGE SGAGKTvsaK
Chk M-II        MMthLhEpav LyNLKeRY.a awMIYTYsGl fcVtVNPYkw LP.VYnPEvV laYRGKkrqE aPPHIFsIsD nAYqfMltDr enQSIlItGE SGAGKTvnTK
Consensus       -LMYL-E--L LHNLKVRY-- DK-IYTYVGN -LVAVNPY-- LP-IY-PE-I K-YRGKSLGE -PPHIFAIAD KAY-DMRVD- --QSIIVSGE SGAGKTE-TK

                201                                                                                            300
Pig M-VI        FVLRYLTesY GtG..... .....qdIDD rIvEANPlLE AFGNAKTVRN NNSSRFGKFV EIHFneKsav VGGFVShYLL EKSRIcvQgk EERNYHIFYr
 Dro 95f        YlLkYLcysh dSa..... .....GpIEt kILDANPVLE AFGNAKTcRN NNSSRFGKFI EVHYDAKcqm VGGYIShYLL EKSRIctQsa EERNYHVFYm
Bov BBMI        LVMsTvAsVC GkGEqv.... .....nsVkE qlLqaNPVLE AFGNAKTIRN NNSSRFGKYm DIeFDfKgfp lGGvItnYLL EKSRVvkQLE gERNFHIFYQ
 Mus M-V        YaMRYFATVs GSas..... ....eanVEE kVLasNPIME siGNAKTcRN dNSSRFGKYI EIgFDkryri IGanmrtYLL EKSRVvFQaE EERNYHIFYQ
Chk M-II        rViqYFATIa aSGEkkkeeq sgkmqGtlED qIisANPlLE AFGNAKTVRN dNSSRFGKFI rIHFgAtgkl asadIetYLL EKSRVtFQLp aERsYHIFYQ
Consensus       YV-RY-ATV- GSGE------ -----G--E- -IL-ANPVLE AFGNAKTVRN NNSSRFGKFI EIHFDAK--- VGG-IS-YLL EKSRV-FQLE EERNYHIFYQ

                301                                                                                            400
Pig M-VI        LcAGA..sEd iRErLhLssP DnFrYLnrGC TrYFANkeTD kqIlqnrKSp eylkaGSLKd PllDDhgDPi rMctAMkkiG LdDEEKLdlF RVVAgVLHLG
 Dro 95f        LlAGA..Pqq lRDKLsLGkP DDYrYLs.GC TqYFANakTE qlIpgsqKSk nhqqkGpLKd PiIDDyqhFh nLDkALgrLG LSDtEKLGIY slVAaVLHLG
Bov BBMI        LlAGAd.aqL lkaLklerdt ggYaYLnpd. .......... .......... .....tS.rV dGmDDdanFk vLqSAMTViG PSDEEirqVL eVaAlVLkLG
 Mus M-V        LcAsAKlPEP .kMLrLrGNs DnFhYtkQG. .......... .......... .....GSpmI eGVDDakEMa htrqAcTlLG iSEsyqMGIF RIlAgILHLG
Chk M-II        i.msnKkPEL idmLLitNP yDYhYvsQG. .......... .......... .....eit.V PsIDDqeELm atDSAidILG FSaDEKtaIY kltgaVMHyG
Consensus       L-AGAK-PEL -R-LL-LGNP DDY-YL-QGC T-YFAN--T- --I----KS- -----GSLKV PGIDD---EP- -LDSAMT-LG -SDEEKLGI- RVVA-VLHLG

                401                                  Phosphorylation Site ?                                     500
Pig M-VI        NIDFEE.agS tsGgCnlknk sTqaLEycAE kLLGLDqDDL RvsLttRVMl TtsgGsKdIV IkvPLKVeQA nNARDALAKt VYShLFdhVV NRVNQcFPFe
 Dro 95f        NIsFEEipdd vrGgCQvsea seqmLtItsg .LLGvDqtEL RtALvSRVMq skggGfKdIV ImvPLKIyaA sNARDALAKA IYSrLFdRIV gLINQSiPFq
Bov BBMI        NVELineFqa ngvpasgIrD grgVqEI.gE .LvGLnasvEL erALCSRtMe TAkE.....k VVttLnViQA qyARDALAKn IYSrLFnWlV NRINeSikvg
 Mus M-V        NV....gFaS rdsdsctIPp khepLtIfcD .LMGvDyEEM chwLChRkLa TAtE.....t yIkPisklQA tNARDALAKh IYskLFnWIV dhVNQaLh.s
Chk M-II        Nl....kFkq kqreeQeaPD gTeVaDkaAy .LMGLnsaEL lkALCyprvk vgnE.....f VtkgqtVsQv hNsvgALAKA VYskMFlWmV iRINQqLd.t
Consensus       N--FEE-P-S --G-CQ-IPD -T-VLEI-AE -L-GLD--EL R-ALCSRVM- TA-EG-KGTV ---PLKV-QA -NARDALAKA IYS-LF-WIV NRINQ5LPF-

                501                                                                                            600
Pig M-VI        tSs..YFIGV LDIAGPEyPE hNSFEQFCIN YCNEKLQQFF NErILKeEQE lYqXEGlgvn eVhYv.DNQD CIDLIEArlv GILDiLDSEn rLPq.PSDqh
 Dro 95f        aSn..FYIGV LDIAGFEyPt vNSFEQFCIN YCNEKLQkFF NDnILKnEQE lYKrEGlnvp eItFt.DNQD iIELIEAKsn GIFtLLDEEs kLPK.P8ySh
Bov BBMI        tgekrkvmGV LDIyGFEiLE dNSFEQFvIN YCNEKLQQvF iEmtLKeEQE EYKrEGIFWv kVEY.FDNgi icnLIEknqr GILaMLDEEC lrPgvvSDSt
 Mus M-V        avkqhsFIGV LDIyGFEtFE INSFEQFCIN YaNEKLQQqF NmEVFKlEQE EYmKEqIFWt lIDF.YDNQp CInLIE.skL GILDLLDEEC kMPK.gtDdt
Chk M-II        kqprqYFIGV LDIAGFEiFD fNSFEQLCIN FtNEKLQQFF NhHmFvlEQE EYKKEGIeWe fIDFgmDlaa CIELIE.KpM GIFsiLEEEC mFPK.atDts
Consensus       -S---YFIGV LDIAGFE-FE -NSFEQFCIN YCNEKLQQFF NEHILK-EQE EYKKEGIPW- -IDF--DNQD CIELIEAK-- GILDLLDEEC -LPK-PSDS-

                601                                                                                            700
Pig M-VI        FTs...agHq khkdHFrLsI PRkSKlaiHR niAyDEGFII RHFAGaVcYe ttqFVEKNND ALhmsLesLi ceSRdKFiRE LFesstnn.. ..........
 Dro 95f        FTA...svHk SwaNHYrLgl PRsSrlXaHR tLrdEGFlV RHFAGaVcYN tEqFIEKNND ALhasLeGLv QecdnpLLqt LFPsgsst.. ..........
Bov BBMI        FlAKL.nQlF SKhsHYeskV tqneqrqydh sMg.lscFrI cHYAGkVtYN vnsFIDKNND lLfrdLsqam wkaRhpLLRs LFPe..... ..........
 Mus M-V        WaqKLYntHL nKcalFe... ....Kpr... .Ms.nkaFII kHFAdkVeYq cEgFlEKNkD tvfEeqikvl kaSkfKMLpE LFqddakais ptsatssGrt
Chk M-II        FknKLYdQHL gKsNnFq... ....KpKpak gkA.EahFsl vHYAGtVdYN isgWlEKNkD pLnEtviGLy QkSsvKtLal LFatyggeae ggggkkgGk.
Consensus       FTAKLY-QHL SK-NHF-L-- PR-SK-K-HR -MA-EEGFII RHFAG-V-YN -E-FIEKNND AL-E-L-GL- Q-SR-KLLRE LFP------- -------G--

                701                                  Actin Binding Site                                        800
Pig M-VI        .........n KdtkqkAGKL sFIsVGnkFK TqLNLLLDkL RSTGasFIRC IKPNlKmTsh HFEGaqILsQ LqCSGmvsVl dlMqgGFPSR asFhEvYnmY
 Dro 95f        .........s vr.....GKL nFIsVGskFK TqLgeLMEKL eqnGtnFIRC IKFNsKmidr qFEGsLaLaQ LkCSGtisVl elMshGYPSR VLFADLYSmY
Bov BBMI        .........  .GdPkQAslk rppTaGAqrK ssvttLMknL ySknPnYIRC IKPNEhqqrG HFsfELVsvQ aqYlGlLsnV RVrRaGYayR qaYgsFLeRY
 Mus M-V        pltrvpvkpt KGrPpQtaKs hkkTVChqFr nsLhLLMEtL naTtPhYVRC IKPNDfkfPf tFDekravqQ LracGvLsEI RIsarGFPSR wtYqEFFSRY
Chk M-II        .........k KGssfQ.... ...TVsAlFr enLNkLManL RSThPhFVRC IiPNEtkTPG amShELVLhQ LrCnGvLsgI RIcRkGFPSR VLYADFkqRY
Consensus       ---------- KG-P-QAGKL -FITVGA-FK T-LNLLME-L RSTGP-FIRC IKPNEK-TPG HFEGELVL-Q L-CSG-LEVI RIMR-GFPSR VLYA-FYSRY

                801
Pig M-VI        k...kSLPdk LarLDprlFC kaLFkaLgLn eiDYKFGlTK VFFR
 Dro 95f        k...svLPPe LVsLpartFC EaMFgSLnLs akDFKPGiTK VFFR
Bov BBMI        RlLsrStwPr wnggD.qegv EkvLGeLsMs sEELaFGKTK IFiR
 Mus M-V        RVL...Mkqk dVLgDrkqtC knvLekLiLd kDkYqFGKTK IFFR
Chk M-II        RVLnaSaiPe gqFMDakkas EkLLGSidvd htqYrPGhTK VFFk
Consensus       RVL--SLPP- LV-LD---FC E-LLGSL-L- --DYKFGKTK VFFR
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C

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Pig M-VI    755 TKVFFRPGKF AEFDQIMKSD PDHLAELVKR VNHWLICSRW KKVQWCSLSV
Dro 95f     749 TKVFFRPGKF VEFDRIMRSD PENMLAIVAK VKKWLIRSRW VKSALGALCV
Bov BBMI    676 TKIFIRSPKT LFYLEEQRRL RLQQLATLIQ KTYRGWRCRT HYQ...LMRK
Mus M-V     746 TKIFFRAGQ. VAYLEKLRAD KLRAACIRIQ KTIRGWLLRK RYL...CMQR
Chk M-II    763 TKVFFKAGL. LGLLEEMRDD KLAEIITRTQ ARCRGFLMRV EYRRMVERRE

Pig M-VI    805 IKLKNKIKYR AEACIKMQKT IRMWLCKRRH KPRIDGL...
Dro 95f     799 IKLRNRIIYR NKCVLIAQRI ARGFLARKQH RPRYQGI...
Bov BBMI    723 SQIVISSWFR GNMQKKH..Y RKMKASALLI QAFVRGWKAR
Mus M-V     792 AAITVQRYVR GYQARCYAKF LRRTKAATTI QKYWRMYVVR
Chk M-II    812 SIFCIQYNVR SFMNVKHWPW MKLFFKIKPL LKSAE.....
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D

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                    .          .          .          .          .
Pig M-VI    1035 LRRGPAVQATKAAAGTKKYDLSKWKYAELRDTINTSCDIELLAACREEFH
                 | |:..:.| ..| |..||||||||||.||||.||||||||||.|||:|||
 Dro 95f    1045 LIRSENLRAQQQALGKQKYDLSKWKYSELRDAINTSCDIELLEACRQEFH
                    .          .          .          .          .
Pig M-VI    1085 RRLKVYHAWKSKNKKRNT.ETEQRAPKSVTDYAQQNPAVQLPARQQEIEM
                 ||||||||||.||:||.| :.::|||.|| : | ..|:: |   ||| :
 Dro 95f    1095 RRLKVYHAWKAKNRKRTTMDENERAPPSVMEAAFKQPPLVQPI..QEI.V
                    .          .          .          .          .
Pig M-VI    1134 NRQQRFFRIPFIRSADQYKDPQNKKKGWWYAHFDGPWIARQMELHPDKPP
                 . |:|:|||||:|.    ..|:|.|:|:|||||||.||||||||:||||
 Dro 95f    1142 TAQHRYFRIPFMRA....NAPDNTKRGLWYAHFDGQWIARQMELHADKPP
                    .          .          .          .          .
Pig M-VI    1184 ILLVAGKDDMEMCELNLEETGLTRKRGAEILPRQFEEIWERCGGIQYLQN
                 ||||||.|||:||||.|||||||||||||||.::|:  ||| || .| .
 Dro 95f    1188 ILLVAGTDDMQMCELSLEETGLTRKRGAEILEHEFNREWERNGGKAYKNL
                    .
Pig M-VI    1234 AIESRQARPTYATAMLQN 1251
                 :    .|:|. :.| :|.
 Dro 95f    1238 G....AAKPNGPAAAMQK 1251
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Figure 2. Similarities between porcine myosin-VI and other myosin heavy chains. (*A*) Schematic of the predicted structure of class VI myosins. The stippled box represents the ~750 aa homologous to other myosin motors (*HEAD*). The light chain binding or neck domain is divided into the "50-aa" linker domain and the "IQ" motif. The tail portion is divided into a coiled-coil (*CC*) domain, marked by a zig-zag–filled box and a unique globular region (*TAIL*). (*B*) Alignment of myosin-VI motor domains to the motor domains of myosins-I, -II, and -V. Using the Pileup and Pretty programs of the Wisconsin package (Devereaux et al., 1984), the head sequence of porcine myosin-VI (*Pig M-VI*) is compared to the head sequences of *Drosophila* myosin-VI 95F MHC (*Dro 95F*; Kellerman and Miller, 1992), bovine brush border myosin-I (*Bov BBMI*; Hoshimaru and Nakanishi, 1987), mouse dilute myosin-V (*Mus M-V*; Mercer et al., 1991), and chicken pectoralis muscle myosin-II (*Chk M-II*; Maita et al., 1991). Below the alignment is a consensus sequence. In this consensus, capital letters indicate identity in two or more myosins. Alignments begin with the first amino acid of the protein sequence of chicken muscle M-II to allow comparison with the three-dimensional structure of this motor (Rayment et al., 1993*b*). The end of the head was defined as the last consensus sequence before the light chain binding motifs, TKVFFR. Above the alignment, the conserved ATP-binding and actin-binding sites are marked (Walker et al., 1982; Warrick and Spudich, 1987). The sequence phosphorylated in amoeboid myosins is boxed (Brzeska et al., 1989). (*C*) Comparison of the location of light chain binding motifs between myosins-VI and myosins-I, -II, and -V. Beginning with the final head consensus sequence TKVFFR, sequences for myosins-I, -II, and -V were aligned to allow for colocation of the light chain binding motifs as defined by Cheney and Mooseker (1992) and detailed by the three-dimensional structure of chicken skeletal M-II S1 fragment (Rayment et al., 1993*b*). These IQ motifs are boxed. The precise amino acid numbers are shown to the left of each line. Above this alignment, the amino acid sequence of porcine myosin-VI and *Drosophila* 95F MHC are shown for comparison. These myosins-VI harbor only a single "IQ" motif ~50 aa downstream from the end of the head (boxed). Alignment algorithms pair this single motif with the third IQ motif of myosins-I and -V by introducing gaps in the myosin-VI sequence, but it is equally related to all light chain binding motifs (not shown). (*D*) Alignment of the tail domains of porcine myosin-VI and *Drosophila* 95F MHC. Tail sequences beginning with the first amino acids following the predicted coiled-coil domain were compared using the Bestfit program from the Wisconsin package. Precise amino acid numbers are shown to the left of each line. Within the alignment, a dash indicates identity while dots indicate degree of similarity.

Immunoprecipitations

The ultra speed supernatant from the LLC-PK_1 cell homogenization was used as the source of myosin-VI for these experiments. Before use, the extract was precleared by incubation with protein A-Sepharose CL-4B beads (Pharmacia LKB Biochemicals; reconstituted in buffer A) for 1 h at 4°C. Approximately 200 μl of this cleared extract was mixed with 20 μg of affinity-purified rabbit anti-myosin-IV antibody, and it was allowed to mix overnight at 4°C. 3 mg protein A-Sepharose beads was added to the mix for 1 h, and the beads were washed three times with buffer A before resuspending the pellet in sample buffer and boiling.

Cosedimentation Assays

The ultra speed supernatant from the homogenization of LLC-PK_1 cells in buffer A was concentrated fivefold using a Centricon-30 microconcentrator (Amicon Inc., Beverly, MA) before use in actin-binding assays. F-actin (6 μM) was added to aliquots in either the presence or the absence of 10 mM ATP, and was allowed to incubate at room temperature for 20 min before being spun at 100,000 *g* for 20 min. F-actin alone controls were included. Pellets and supernatants were collected, brought up to equivalent volumes, and processed for gel electrophoresis.

Indirect Immunofluorescence of Cultured Cells

Coverslip-grown LLC-PK_1 cells were processed for indirect immunofluorescence in six-well plates essentially as described by Bement et al. (1993). Briefly, cells were washed twice with 37°C PBS before fixation for 30 min in room temperature 4% paraformaldelyde in PBS/50 mM EGTA. Cells were permeabilized by dipping in −20°C acetone, then rehydrated in PBS-EGTA. Cells were then rinsed in PBS-EGTA/1% BSA and blocked for 20 min in PBS-EGTA/10% BSA before incubations with antibodies. Affinity-purified rabbit anti-myosin-VI was used at a concentration of 10–40 μg/ml. Fluorescein-phalloidin was used at a concentration of 120 nM (Molecular Probes, Inc., Eugene, OR) and added with the rhodamine-conjugated goat anti-rabbit secondary antibody. Fluorescence images of subconfluent LLC-PK_1 cells were obtained as described by Forscher and Smith (1988). Imaging of confluent cells was done using a laser scanning confocal microscope (MRC600; Bio Rad Labs, Hercules, CA).

Immunohistochemistry

Mouse kidney was dissected, minced, and fixed for 5 min on ice in 4% formaldehyde in PBS/EGTA. Tissue was rinsed in PBS-EGTA, quenched for 10 min in 0.05% sodium borohydride, rinsed, and then cryoprotected in 1 M sucrose before embedding in OCT media (Miles Inc., Elkhart, IN) and freezing in liquid nitrogen-cooled isopentane. 4-μm frozen sections were cut and applied to slides coated with Vectabond (Vector Labs, Burlingame, CA) and stored at −20°C until used. The OCT was removed by immersing slides in −20°C acetone, and rehydrated in water. Anti-myosin-VI antibody was added at a concentration of 10 μg/ml in PBS-EGTA with 0.1% BSA for 20 min. Nonimmune IgG was used as a negative control. After antibody incubation, the slide was washed three times in PBS-EGTA, and the FITC-conjugated secondary and rhodamine phalloidin were applied for 20 min. Slides were again washed and then observed with a light microscope equipped for epifluorescence (Carl Zeiss, Inc., Thornwood, NY).

Results

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

To initiate our study of the multiple unconventional myosins expressed by LLC-PK_1 cells, one of the DNA fragments identified by PCR as a putative myosin (Bement et al., 1994*a*) was used to screen a λZAP 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 NH_2-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., 1993*b*), and for this reason we named this LLC-PK_1-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., 1993*b*), 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 (CCAAAATGG) is consistent with the eukaryotic start site consensus sequence (CC $^{A}/_{G}$CCATGG; 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.

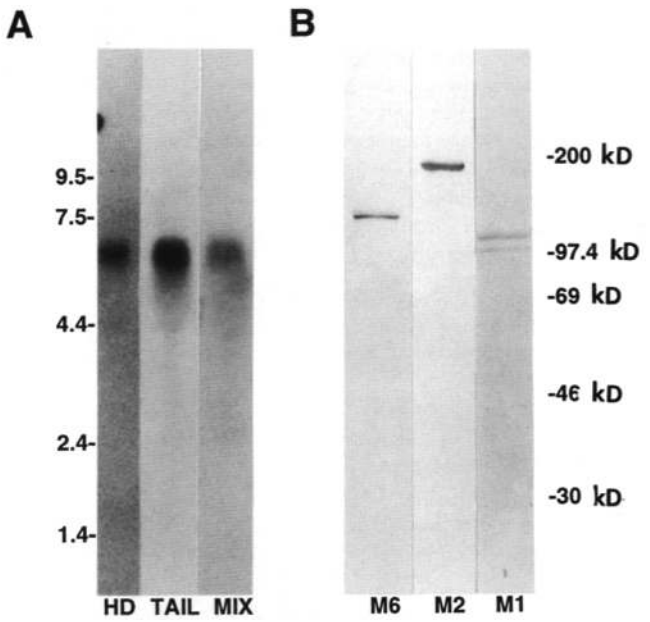

Figure 3. Expression of porcine myosin-VI in LLC-PK_1 cells. (*A*) Northern blot analysis using head and tail myosin-VI probes. Individual lanes containing LLC-PK_1 cell RNA were probed with either a ^{32}P-labeled, 900-bp NH_2-terminal SacI fragment (*HD*) or a 575-bp COOH-terminal SacI fragment (*TAIL*) 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-PK_1 cells (for M6 and M2) or 5.4×10^5 LLC-PK_1 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.

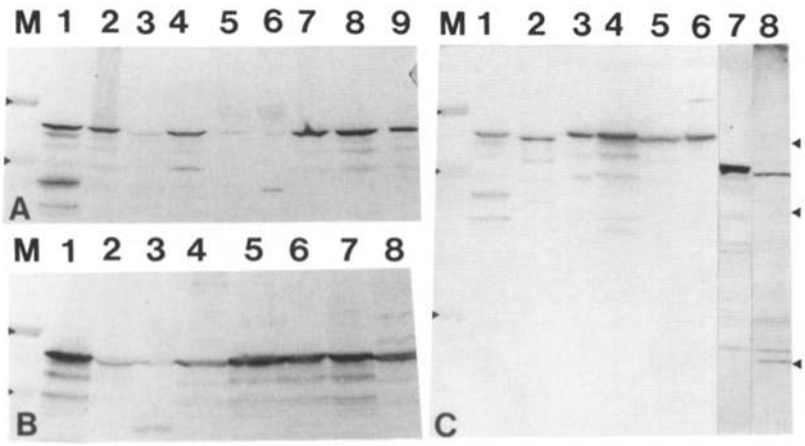

Figure 4. Myosin-VI expression in pig and rat tissues and in other organisms. (*A*) Detection of myosin-VI in pig tissues by immunoblotting. Total cell extracts from pig tissues were separated on a 5–16% polyacrylamide gel and blotted with the affinity-purified rabbit anti–myosin-VI antibody. Approximately equal amounts of protein were loaded in each lane for each protein source. Whole-cell extract from LLC-PK_1 cells was also included for comparison. The affinity-purifed 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-PK_1 (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_{BBe}; 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 NH_2 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 NH_2 terminus. The affinity-purified antibodies were then used to characterize the expression profile of myosin-VI.

By immunoblot, myosin-VI in LLC-PK_1 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. 3*B*). 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-PK_1 extracts (Fig. 3 *B*). This protein blot analysis provides further evidence that the clonal LLC-PK_1 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-PK_1 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-PK_1 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 colocate with major F-actin–containing structures (Fig. 5). As judged by indirect immunofluoresence, myosin-VI was not found associated with cell–cell junctions in confluent monolayers (Fig. 5, *a*

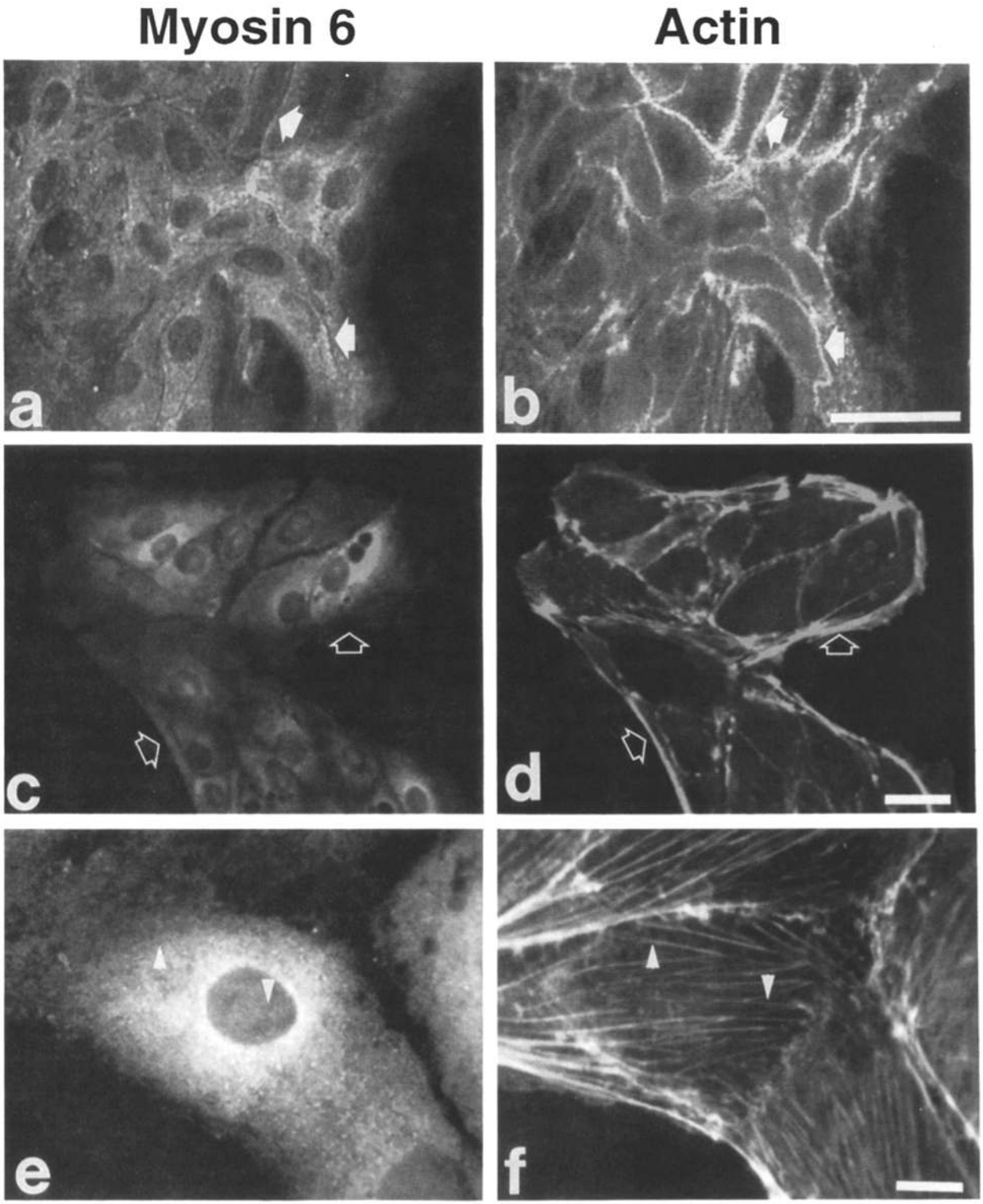

Figure 5. Localization of myosin-VI in undifferentiated LLC-PK$_1$ cells. Confluent (*a–b*) and subconfluent (*c–f*) LLC-PK$_1$ cells were grown on coverslips, fixed with paraformaldehyde, and subjected to indirect immunofluorescence with 40 μg/ml affinity-purified rabbit anti-myosin-VI tail antibodies (*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*.

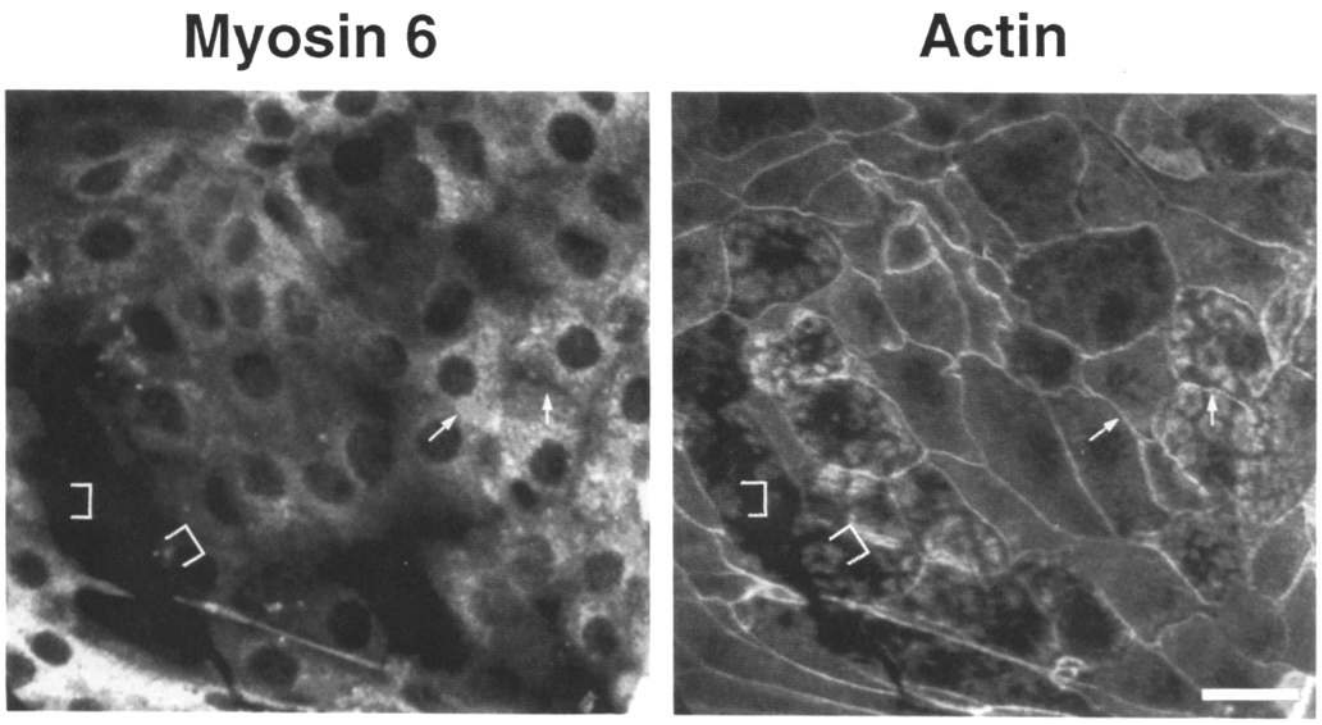

Figure 6. Localization of myosin-VI and actin in differentiated LLC-PK$_1$ cells. Coverslip-grown LLC-PK$_1$ cells 13 d after confluence were prepared for confocal immunofluorescence microscopy as described in Fig. 5. An optical section through the apical domain of the monolayer at the level of the tight 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 μm.

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-PK$_1$ cells. Rather, myosin-VI appeared as a diffuse, punctate stain within the cytoplasm of LLC-PK$_1$ cells. A similar staining pattern was also observed in differentiated CaCo-2$_{BBe}$ 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-PK$_1$ cell line. Approximately 2 wk after the initiation of cell–cell contact, LLC-PK$_1$ (*CL4*) 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-PK$_1$ 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-PK$_1$ cells were homogenized and fractionated by differential velocity sedimentation (Fig. 7 *A*). Visual inspection indicated that fractionation of LLC-PK$_1$ 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 supernatant. 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-PK$_1$ 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 ~0.8% of total protein in LLC-PK$_1$ 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-PK$_1$ 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

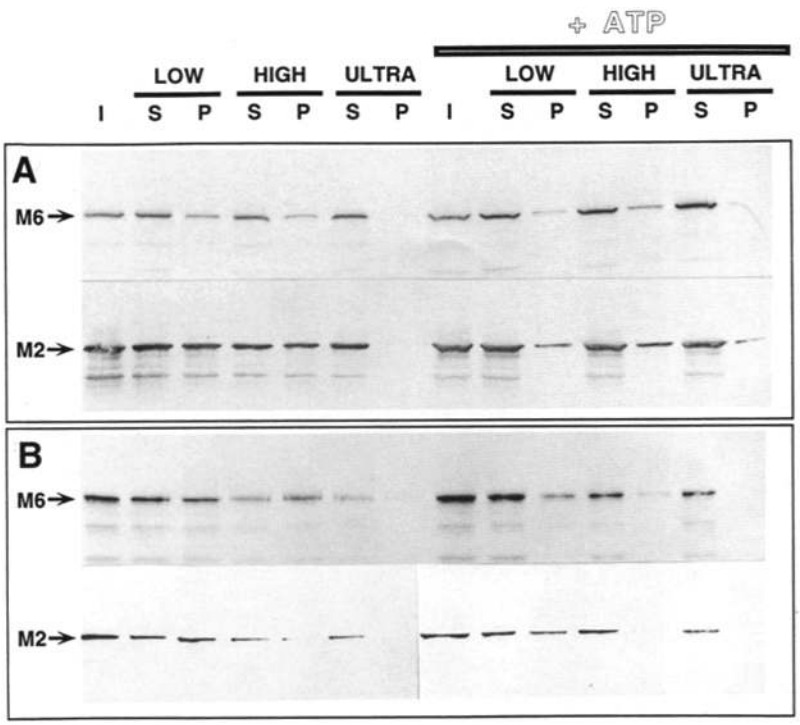

Figure 7. Myosin-VI exhibits a different subcellular fractionation pattern between LLC-PK$_1$ cells and kidney. (*A*) LLC-PK$_1$ cells or (*B*) whole mouse kidneys were homogenized and fractionated by differential sedimentation. The initial homogenization was done in the presence or absence of 10 mM 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-PK$_1$ 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-PK$_1$ 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

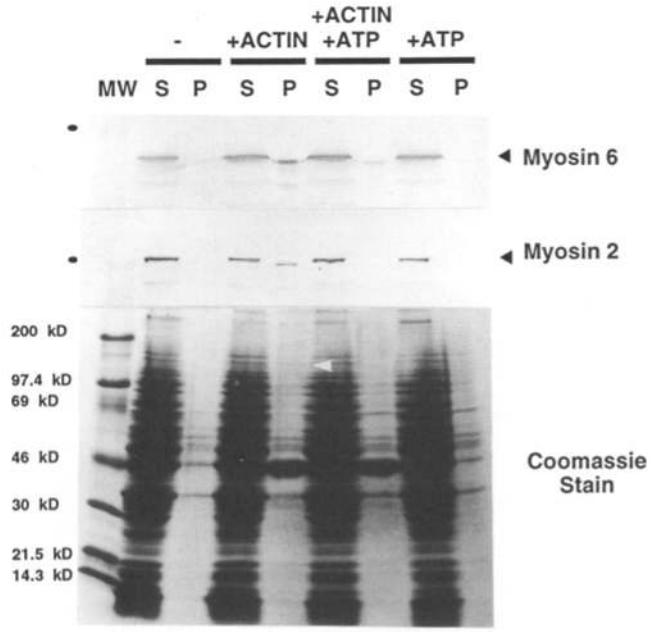

Figure 8. Myosin-VI binds F-actin in an ATP-sensitive fashion in cosedimentation assays. The ultra speed supernatant from fractionated LLC-PK$_1$ cells (Fig. 7) was incubated with F-actin, 10 mM ATP, or both F-actin and ATP and spun at 100,000 *g*. The resulting supernatants (*S*) and pellets (*P*) were 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.

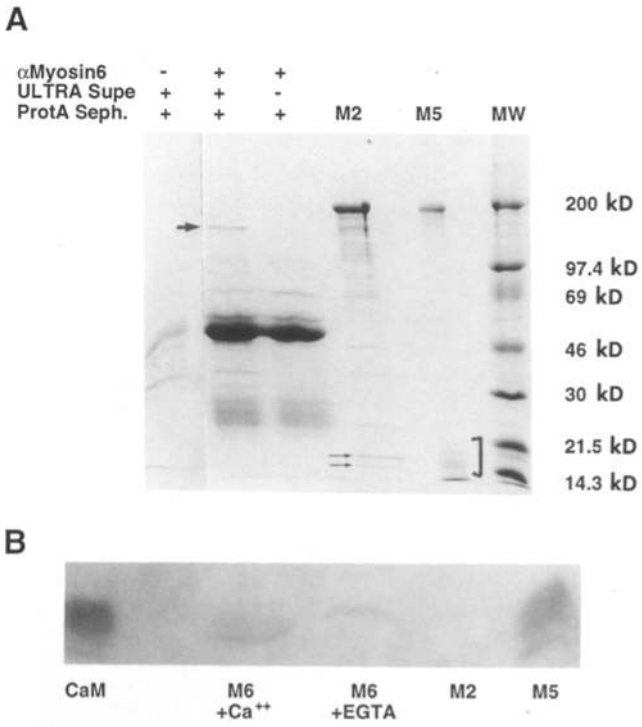

Figure 9. Myosin-VI is associated with a calmodulin light chain. (*A*) Coomassie-stained polyacrylamide gel of protein samples used for anti-calmodulin protein blots. Myosin-VI was immunoprecipitated from the ultra speed supernatant of fractionated LLC-PK$_1$ cells using 10 μg affinity-purified rabbit 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 μg purified chicken brush border myosin-II (*M2*) and 1 μg 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 μg purified bovine brain calmodulin was included as a positive control (*CaM*). 3 μg 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).

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-PK$_1$ 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-PK$_1$ cell line (Fig. 7 *B*). Unlike the myosin-VI within the LLC-PK$_1$ 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-

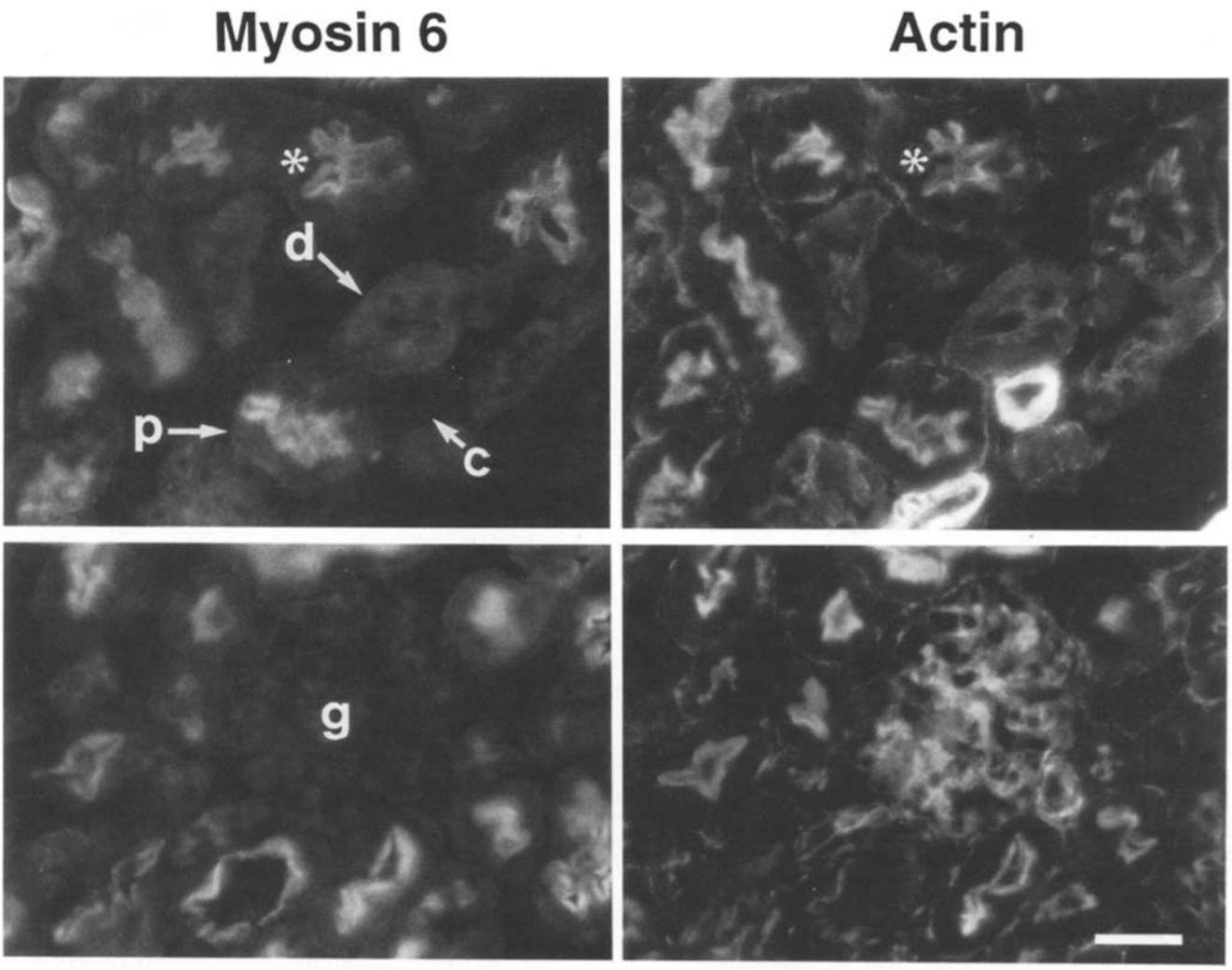

Figure 10. Indirect immunofluorescence localization of myosin-VI and actin within the kidney. Frozen thin sections of formaldehyde-fixed adult mouse kidney were incubated with 10 μg/ml affinity-puriifed rabbit anti–myosin-VI antibodies and were also stained with fluorescein-labeled phalloidin to visualize 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-PK_1 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-PK$_1$ cells identified nine different myosins, but only one myosin-I–like clone (Bement et al., 1994*a*). 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-PK$_1$ 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-PK$_1$ 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^+ exhange (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-PK$_1$ 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-PK$_1$ 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-PK$_1$ 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-PK$_1$ 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 signal is required to initiate the assembly of this myosin-I into the brush border.

Porcine M6	SLTTRVMLTTAGGAKGTVIKPLLKVEQANNARDALAK	426
Dro 95F	ALVSRVMQSKGGGFKGTVIMVPLKIYEASNARDALAK	425
Aca M1A	AGTTYALNLNKMQAIGSRDALAK	N.D.
Aca M1B	LLFRVLNTGGAGAKKMSTYNVPQNVEQAASARDALAK	335
Aca M1C	LLYRTITTGEQGRGRSSVYSCPQDPLGAIYSRDALSK	331
Dict M1A	SSLVSRQISTGQGARISTYSVPQTVEQAMYARDAFAK	354
Dict M1B	FRVINTGGAGGAGNRRSTYNVPQNVEQANGTRDALAR	352
Dict M1D	CFRTISTGTQGRSARVSTYACPQNSEGAYYSRDALAK	352
Dict M1E	IALCYRSISTGVGKRCSVISVPNDCNQAAYSRDALAK	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.

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-PK$_1$ 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. (1994*b*), 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., 1993*a*; 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 cosedimentation 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., 1993*a*). 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., 1993*a*; 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-PK_1 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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