Primary Structure and Cellular Localization of Chicken Brain Myosin-V (p190), an Unconventional Myosin with Calmodulin Light Chains

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Abstract. Recent biochemical studies of p190, a calmodulin (CM)-binding protein purified from vertebrate brain, have demonstrated that this protein, purified as a complex with bound CM, shares a number of properties with myosins (Espindola, F. S., E. M. Espreafico, M. V. Coelho, A. R. Martins, F. R. C. Costa, M. S. Mooseker, and R. E. Larson. 1992. *J. Cell Biol.* 118:359–368). To determine whether or not p190 was a member of the myosin family of proteins, a set of overlapping cDNAs encoding the full-length protein sequence of chicken brain p190 was isolated and sequenced. Verification that the deduced primary structure was that of p190 was demonstrated through microsequence analysis of a cyanogen bromide peptide generated from chick brain p190. The deduced primary structure of chicken brain p190 revealed that this 1,830-amino acid (aa) 212,509-D) protein is a member of a novel structural class of unconventional myosins that includes the gene products encoded by the *dilute* locus of mouse and the MYO2 gene of *Saccharomyces cerevisiae*. We have named the p190-CM complex "myosin-V" based on the results of a detailed sequence comparison of the head domains of 29 myosin heavy chains (hc), which has revealed that this myosin, based on head structure, is the fifth of six distinct structural classes of myosin to be described thus far. Like the presumed products of the mouse *dilute* and yeast MYO2 genes, the head domain of chicken myosin-V hc (aa 1–764) is linked to a "neck" domain (aa 765–909) consisting of six tandem repeats of an ~23-aa "IQ-motif." All known myosins contain at least one such motif at their head–tail junctions; these IQ-motifs may function as calmodulin or light chain binding sites. The tail domain of chicken myosin-V consists of an initial 511 aa predicted to form several segments of coiled-coil α helix followed by a terminal 410-aa globular domain (aa, 1,421–1,830). Interestingly, a portion of the tail domain (aa, 1,094–1,830) shares 58% amino acid sequence identity with a 723-aa protein from mouse brain reported to be a glutamic acid decarboxylase. The neck region of chicken myosin-V, which contains the IQ-motifs, was demonstrated to contain the binding sites for CM by analyzing CM binding to bacterially expressed fusion proteins containing the head, neck, and tail domains. Immunolocalization of myosin-V in brain and in cultured cells revealed an unusual distribution for this myosin in both neurons and nonneuronal cells. Myosin-V staining was particularly intense in the cell bodies and dendrites of Purkinje cells. Double labeling with wheat germ agglutinin revealed colocalization of myosin-V with cytoplasmic, presumably Golgi-derived, membranes. In primary cultures of neurons and glia, myosin-V immunoreactivity had a punctate distribution more abundant in the region of the Golgi complex and at the tips of long processes such as growth cones. These results, together with the phenotypes of mutations described for the *dilute* and myo2 genes, suggest that the myosin-V family of unconventional myosins may be in part associated with cytoplasmic organelles.

EVIDENCE continues to emerge for the existence of a superfamily of myosin heavy chain (hc)[1] genes encoding, in a given organism, a structurally diverse set of actin-based mechanoenzymes (for reviews see Pollard et al., 1991; Hammer, 1991; Cheney and Mooseker, 1992). The information base for such a superfamily has been best defined for the ameboid organisms, *Acanthamoeba castellani* and *Dictyostelium discoideum*, in which extensive protein characterizations as well as primary structure analyses have been performed. In addition to a single conventional myosin (myosin-II) gene, both these organisms possess mul-

1. *Abbreviations used in this paper*: aa, amino acid; BB, brush border; CM, calmodulin; CNBr, cyanogen bromide; GAD, glutamic acid decarboxylase; hc, heavy chain; MBP, maltose binding protein; nt, nucleotide; pBS, bluescript plasmid; WGA, wheat germ agglutinin.

tiple unconventional myosins, most of which have been structurally defined as myosins-I (Hammer, 1991). For example, five myosin-I genes have been cloned from *Dictyostelium* thus far (Hammer, 1991). Myosins-I have been, to date, operationally defined as having a single-head domain similar in primary structure to that of conventional myosins, but lack the coiled-coil α-helical tail responsible for dimerization and filament formation in the conventional myosins. The tail domains of the ameboid myosins-I are segmented into discrete functional domains that can include sites for membrane binding and ATP-insensitive actin binding.

The existence of unconventional myosins in vertebrate species was established with the characterization of a chicken myosin-I purified from the brush border (BB) of intestinal epithelial cells (for reviews see Pollard et al., 1991; Mooseker et al., 1991); this same myosin-I may be expressed in rat renal BBs as well (Coluccio, 1991). A second myosin-I, with biochemical properties similar to chicken BB myosin-I, has been purified from bovine adrenal medulla (Barylko et al., 1992). Interestingly, this myosin-I, like chicken BB myosin-I, contains multiple calmodulin (CM) light chains. Most recently, Espindola et al. (1992) have characterized a CM-binding protein whose biochemical properties suggest it may be an unconventional myosin. This protein, termed p190 based on its apparent molecular mass by SDS-PAGE, was originally characterized as a CM-binding protein present as a minor component of preparations of actomyosin prepared from vertebrate brain (Larson et al., 1988, 1990).

Like the vertebrate myosins-I described above, p190 is purified as a stable complex with CM in buffers containing EGTA. Although immunologically distinct from conventional brain myosin (Larson et al., 1990; Espindola et al., 1992), the p190-CM complex exhibits a number of properties that suggest that it is a myosin (Espindola et al., 1992). mAbs raised against chicken BB myosin-I, which are reactive with epitopes in the head domain of this myosin (Carboni et al., 1988), bind to p190. The p190–CM complex exhibits ATP-sensitive binding to F-actin. Like myosin, p190–CM exhibits actin-activated MgATPase activity; interestingly, however, maximal actin activation of its MgATPase requires the presence of Ca^{2+} and is further augmented by addition of exogenous CM. Unlike most myosins, the p190–CM complex does not exhibit significant ATPase activity in the presence of K-EDTA.

In the present study, we sought to verify the identification of p190 as a member of the myosin family of actin-based motors by analysis of its primary structure. This was accomplished by isolation and sequence analysis of a set of overlapping cDNAs encoding the full-length protein sequence of chicken brain p190. This analysis revealed that p190 is a member of a novel structural class of unconventional myosins that includes the gene products encoded by the MYO2 gene of *Saccharomyces cerevisiae* (Johnston et al., 1991) and the *dilute* (Mercer et al., 1991) gene of mouse (we term this group of myosins class V since it is the fifth of six such structural classes to be identified; see Discussion). Unlike myosins-I, the tail domains of class V myosins contain segments predicted to form coiled-coil α helices and thus are likely to form dimers. In the present study, an extensive analysis of the domain structure of chicken myosin-V hc was performed, which includes the determination of the region of the chicken myosin-V hc molecule responsible for CM binding. The localization of myosin-V in neuronal and nonneuronal cells and tissues suggest that this myosin is associated, in part, with cytoplasmic organelles. The significance of these findings is discussed with respect to the phenotypes of the mutations described for the MYO2 and *dilute* genes.

Materials and Methods

Isolation of p190 (Chicken Myosin-V hc) cDNAs

Unless specified, molecular cloning methods used were essentially as described by Sambrook et al. (1989). The initial set of p190 cDNA clones was isolated from an adult chicken brain expression library (lambda ZAP vector, oligo dT primed) graciously provided by Douglas Fambrough (Department of Biology, Johns Hopkins University, Baltimore, MD). This library was screened using an IgG fraction from a rabbit polyclonal antisera raised against rat brain p190 (Larson et al., 1990; Espindola et al., 1992). Alkaline phosphatase–conjugated secondary antibody was used for visualization of immunoreactive plaques following the instructions of the supplier (Promega Biotec, Madison, WI). In this initial expression screen, 47 immunoreactive clones were isolated after plaque purification. None of the initial clones extended to the 5′ end of the coding region. Two subsequent hybridization screens were performed to obtain 5′ sequence using polymerase chain reaction products encompassing nucleotides (nt) 2,375–2,846 and 95–512 as probes. Hybridizations were performed in 50% formamide at 42°C using ~0.5–2.5 ng/ml probe DNA and nonfat dry milk as the blocking reagent. Filters were washed at 65°C at a maximal stringency of 0.1× SSC, 0.1% SDS.

DNA Sequencing

Based on size and restriction mapping profiles, several of the largest and apparently overlapping clones were selected for sequence analysis. The cDNA clones were rescued to yield pBluescript SK(−) plasmids following the lambda ZAP in vivo excision protocol (Stratagene Cloning Systems, La Jolla, CA). Both strands of the indicated clones in Fig. 1 were sequenced using either a primer walk strategy or sets of unidirectional ExoIII deletions (Erase-a-Base System; Promega Corp., Madison, WI). Double- or single-stranded sequencing was performed using the dideoxy chain termination method (Sanger et al., 1977), usually using 7-deaza dGTP and T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH).

Sequence Analysis

Sequence entry and analysis was performed using the PC/GENE v6.01 (Intelligenetics, Mountain View, CA) and Genetics Computer Group (GCG; Devereux et al., 1984) suites of computer programs. Multiple sequence alignments were performed using the GCG PileUp program and the default weightings. Values for percentage identity and similarity between sequences were obtained using the GCG BestFit program, and GenBank database searches were performed using the FASTA program of Pearson and Lipman (1988). Prediction of coiled-coil domains was performed using the coiled-coil program of Lupas et al. (1991) with a sliding window of 28 amino acids. A predicted probability of forming a coiled-coil of ≥90% was used as an arbitrary cut-off value for defining segments of coiled-coil structure. PEST sequences were identified by use of the PC/GENE PESTFIND program. The phylogenetic tree of myosin head sequences and the alignment and distance matrix used to generate it were produced using the Clustal V program (Higgins et al., 1992), which uses the neighbor-joining method of Saitou and Nei (1987). For the purposes of this tree, the last amino acid of the myosin head domain was defined as ending at the 18th residue following the "T" in the moderately conserved "TKIFF" sequence that is found near the end of the head in most myosins. This alignment of head sequences thus did not include the myosin neck domain and ended at residue 764 in the p190 sequence or its equivalent in other myosins. The sequences used to generate the tree are available from either the Swissprot or GenBank databases.

Peptide Microsequence Analysis

Myosin-V used for microsequence analysis was purified from chick brain by methods described in Espindola et al. (1992). Final purification before

sequence analysis was performed by electroelution from preparative SDS gels by methods essentially as described in Hunkapillar et al. (1983). Cyanogen bromide (CNBr) cleavage of p190 and electroblotting of the peptides onto polyvinylidene difluoride (Immobilon; Millipore Corp., Bedford, MA) were performed as described by Matsudaira (1987, 1990). The amino-terminal sequence of one of the CNBr-generated peptides was determined at the Protein and Nucleic Acid Chemistry Facility, Yale University.

Generation of Myosin-V hc Head, Neck, and Tail Domain Fusion Proteins

The head, neck, and tail domains of chicken myosin-V hc were subcloned in fusion with maltose binding protein (MBP; 42 kD) in the vector pIH902 (New England Biolabs, Beverly, MA). The subcloning procedures were as follows: For the head domain construct (aa 5–752), the EcoRI and NciI fragment from clone H54 (nt 94–2,337) was blunt-ended with Klenow and ligated into the StuI site of the polylinker region of pIH902. The calculated molecular mass of the head fragment is 86 kD; the fusion protein with MBP was estimated to have a molecular mass of ~130 kD. The neck domain construct was derived from the H11 clone, which was in the reverse orientation in the bluescript plasmid (pBS). The HindIII fragment of this clone (nt 2,360–2,847) was inserted into the HindIII site of pIH902. This neck construct encodes the 20 kD constituting the six putative CM binding sites (aa 760–922), and as a fusion protein had an apparent molecular mass of ~62 kD. The carboxy terminal of this construct ended in frame with the α fragment of β-galactosidase. The tail domain construct was produced by cutting the 8c clone with EcoRI and ligating this fragment (nt 2,778–6,599) into the EcoRI site of pIH902. This tail fragment encodes 109 kD of chicken myosin-V hc (aa 899–1,830), and the fusion protein with MBP was estimated to have a molecular mass of ~151 kD.

The CM binding properties of tissue-purified chicken myosin-V hc and the fusion proteins containing head, neck, and tail domains were assessed by a gel overlay technique (Carlin et al., 1981). Bovine brain CM (Calbiochem-Behring Corp., San Diego, CA) was iodinated in an iodination hood by the lactoperoxidase-enzymobead method according to the manufacturer's instructions using 50 μl of enzymobeads (Bio-Rad Laboratories, Richmond, CA), 1 mg of CM in 200 μl of buffer (100 mM HEPES, 0.5 mM $CaCl_2$, pH 7.2), and 2 mCi of carrier-free $Na^{125}I$. After 0.5 h at room temperature the reaction was quenched by the addition of 50 μl of 10% 2-mercaptoethanol, and the free iodine was removed by the sequential use of two 2.0-ml G-25 fine spin columns (Tuszynski et al., 1980) preequilibrated with the reaction buffer plus 1% 2-mercaptoethanol.

Chicken Myosin-V hc Antibody Production

For production of antibodies reactive with chicken brain myosin-V, a pBluescript (SK−) fusion protein containing most of the tail domain of chicken myosin-V was generated using one of the cDNAs isolated in the initial expression screen (clone 32a in Fig. 1; this clone was not in frame with the β-galactosidase and probably initiated internally at methionine 1057 or 1100); expression was performed in the BB4 strain of *Escherichia coli*. The 90-kD protein encoded by this clone was purified through the following steps, carried out at 4°C: the bacterial pellet was washed in Buffer I (50 mM Tris, pH 7.2, 150 mM NaCl, 10 mM EDTA, 0.2 mM 2-mercaptoethanol, 0.3 mM PMSF); the cells were lysed by sonication in the same buffer containing 1% Triton X-100 and centrifuged at 12,000 *g* for 15 min; the pellet was resuspended by sonication in the same buffer containing 1% Triton X-100 plus 1 M NaCl and 2 M urea, and centrifuged at 12,000 *g* for 30 min. The resulting pellet, which was enriched in the tail domain protein, was resuspended in buffer I and purified by electroelution from preparative SDS gels. Residual SDS was removed by acetone precipitation. The purified protein was used for production of antisera by a commercial service (Pocono Rabbit Farm, Canadensis, PA).

Immunochemical and Cytochemical Techniques

The specificity of the antibodies raised to bacterially expressed protein containing the tail domain of chicken myosin-V hc was assessed by immunoblot analysis (Towbin et al., 1979) against whole rat and chick brain homogenates as well as against myosin-V purified from chick brain by the methods described in Espindola et al. (1992). Immunodetection was carried out using the anti-myosin-V hc serum at a dilution of 1:2,000. Chicken myosin-V hc antibodies were affinity purified by passage of serum over a column made by coupling the bacterially expressed tail domain protein from the 32a clone to CNBr-activated Sepharose.

Localization of myosin-V hc in brain was performed using specimens obtained from rat. Sprague Dawley rats, 175–250 g, were anesthetized and transcardially perfused with ice-cold 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer, pH 7.2. Preparation and immunostaining of frozen sections were performed by the methods of De Camilli et al. (1983). Anti-chicken myosin-V serum was used at a dilution of 1:200; in some experiments, affinity-purified antibodies (at 1–10 μg/ml) were used. At the end of the immunostaining, sections were briefly incubated with FITC-conjugated wheat germ agglutinin (WGA; Vector Laboratories, Burlingame, CA) at a dilution of 1:1,000. Localization of myosin-V hc in primary neuronal cultures was performed using preparations from the hippocampi of 18-d-old fetal rats as described by Banker and Cowan (1977) and Bartlett and Banker (1984). Briefly, hippocampi were dissociated by treatment with trypsin (0.1%, for 15 min at 37°C), followed by vigorous pipetting with a fire-polished Pasteur pipette. Dissociated cells were plated on poly-L-lysine–treated glass coverslips in MEM supplemented with 10% horse serum at densities ranging from 16,000 to 20,000 cells/cm^2. After a

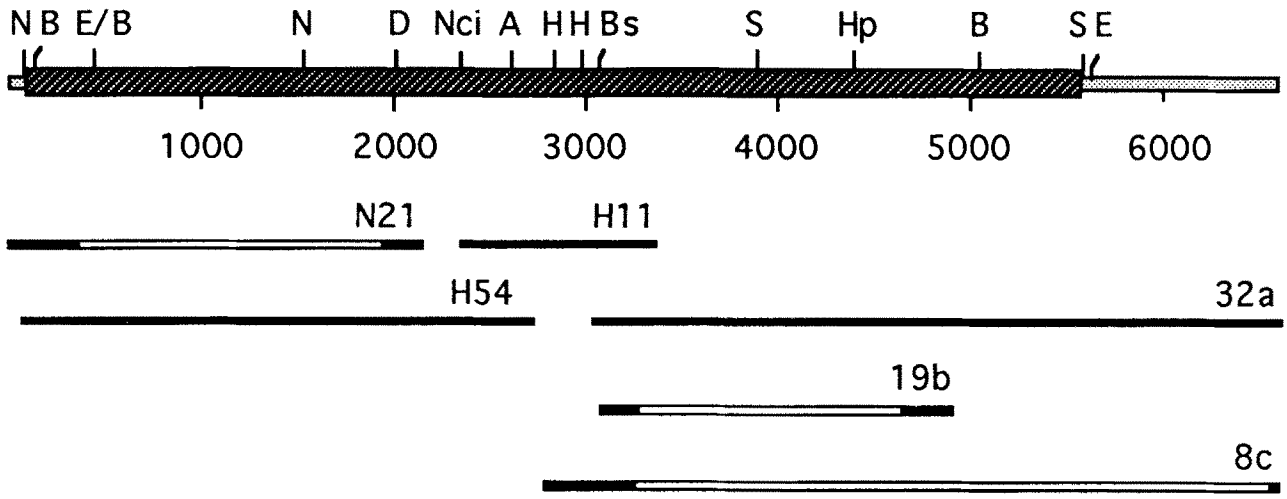
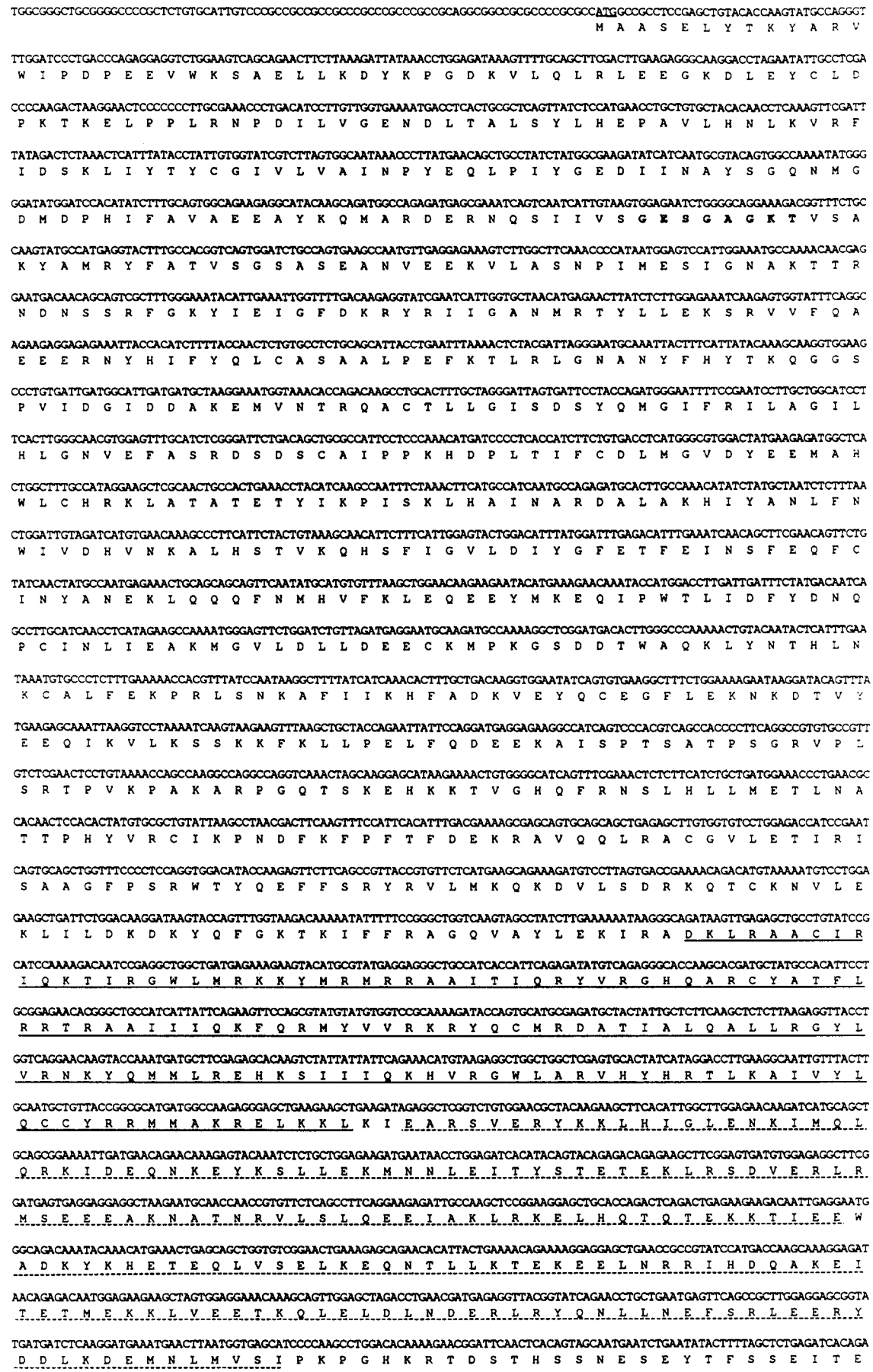


Figure 1. Restriction map and alignment of cDNAs used for sequence determination of chicken brain myosin-V heavy chain. The striped region on the restriction map (nucleotides 83–5,572) represents the open reading frame. The regions of the individual cDNA clones indicated by the solid black shading represent the sequenced portions of the clones. Clone 32a was used to produce, through bacterial expression, tail-domain protein for production of antibodies. Restriction sites: NcoI (*N*), BamHI (*B*), EcoRV (*E*), DraIII (*D*), NciI (*Nci*), AflII (*A*), HindIII (*H*), BspEI (*Bs*), SauI (*S*), and HpaI (*Hp*).



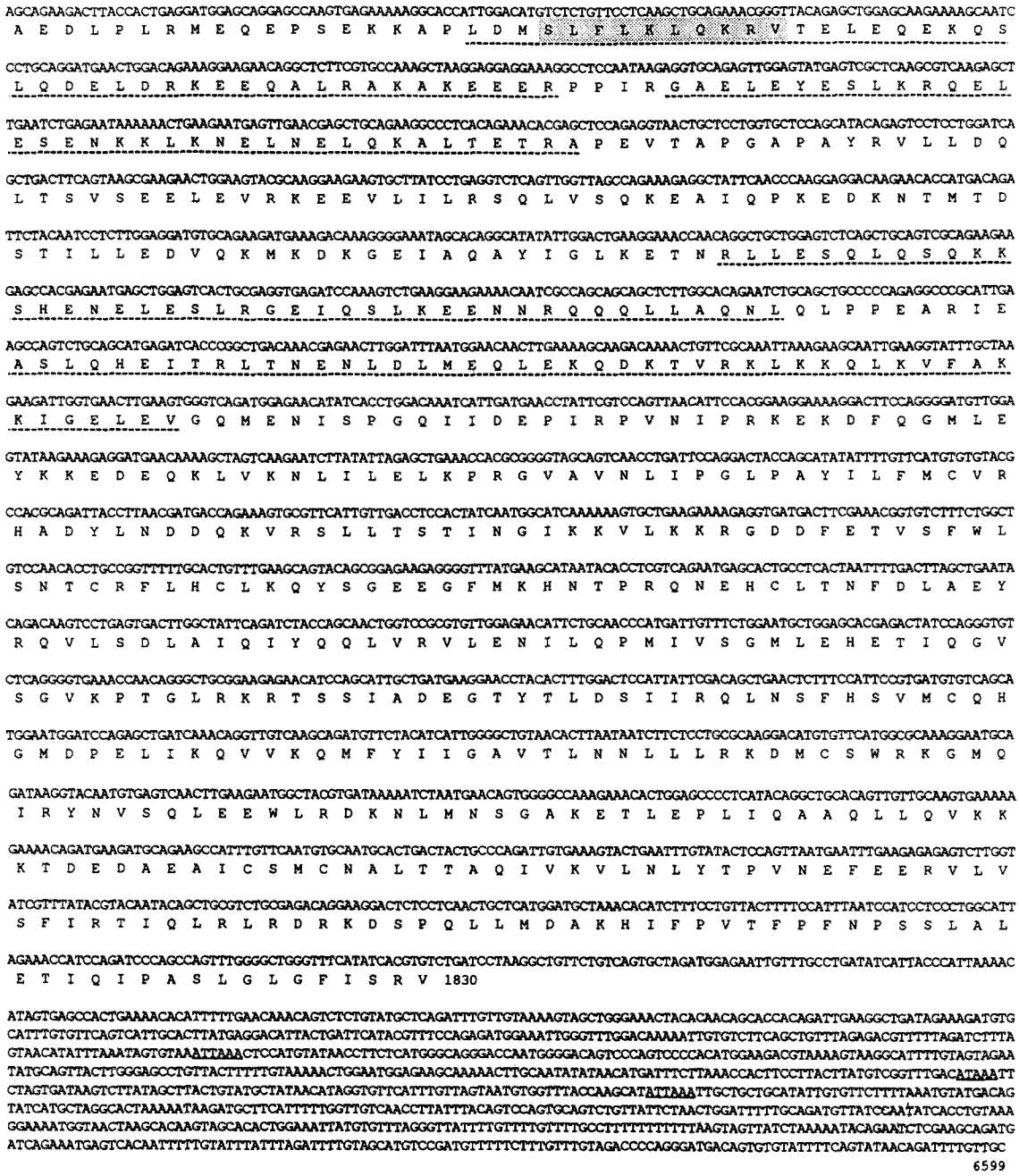


Figure 2. The nucleotide and deduced amino acid sequence of chicken brain myosin-V heavy chain. The initiation codon ATG and potential polyadenylation sites are underlined. The amino acid sequence segment underlined with a solid line denotes the "neck" domain containing the six IQ-motifs; the broken line denotes residues predicted to have a >90% probability of forming a coiled-coil α helix. The shaded sequence matches that determined by microsequence analysis of a CNBr peptide of chick brain myosin-V hc. These sequence data are available from EMBL/GenBank/DDJB under accession number Z11718.

few hours, coverslips were transferred (upside down) to dishes containing a monolayer of cortical glial cells, so that they were suspended over the glial cells but not in contact with them (Bartlett and Banker, 1984). Cells were maintained in MEM without sera, supplemented with 1% HL1 (Ventrex, Portland, ME), 2 mM glutamine, and 1 mg/ml BSA. Neurons were fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer containing 0.12 M sucrose. For immunostaining, coverslips were processed through the following steps: permeabilization and quenching of nonspecific protein binding sites with GSDB (0.3% Triton X-100, 16% goat serum in Buffer A: 500 mM NaCl, 20 mM phosphate buffer, pH 7.4); incubation with anti-myosin-V antibodies (anti-rat or anti-chicken) at a dilution of 1:200 in GSDB; wash in buffer A; incubation with rhodamine-conjugated goat anti-rabbit antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN) at a dilution of 1:100 in GSDB; wash with buffer A followed by PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) incubation with FITC-conjugated phalloidin (Molecular Probes, Inc., Junction City, OR) at a dilution of 1:100 in GSDB; wash with PBS; and final rinse in 5 mM phosphate buffer (De Camilli et al., 1983; Matteoli et al., 1991).

Neuronal cultures and brain sections were mounted in 70% glycerol in PBS containing 1 mg/ml phenylenediamine. Preparations were examined with a Zeiss Axiophot microscope equipped with epifluorescence microscopy and photographed with T-MAX 100 film (Eastman Kodak Co., Rochester, NY).

Other Methods

Glutamic acid decarboxylase (GAD) activity was assessed by trapping in

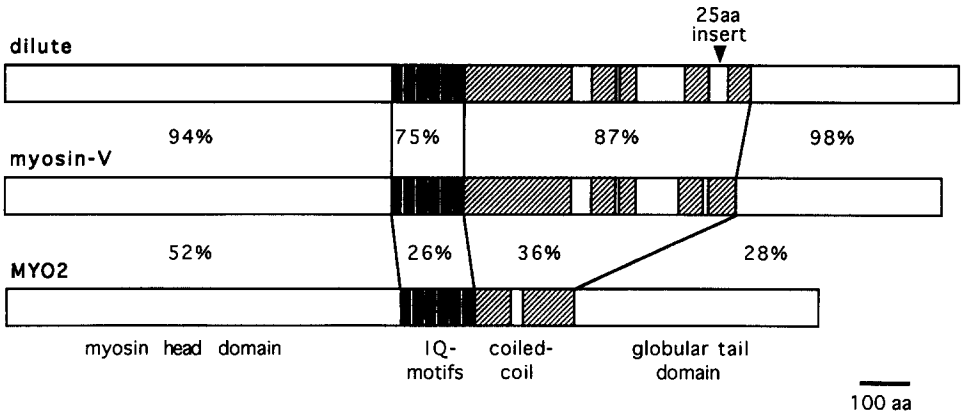


Figure 3. Schematic comparison of the primary structure of chicken myosin-V hc with that of *dilute* and yeast MYO2. Regions predicted to form coiled-coil α helices are indicated by the diagonal hatching. The percent amino acid sequence identities between the various domains are indicated. Note that chicken myosin-V hc and *dilute* align well along their entire lengths except for a 25-aa insert in the tail domain of *dilute*.

filter paper the CO_2 released from [1-^{14}C]L-glutamate and counting the $^{14}CO_2$ in a liquid scintillation counter as described by Baekkeskov et al. (1990).

Results

Deduced Primary Structure of Chicken Brain p190 (Myosin-V hc)

A set of partial length, overlapping cDNAs (clones 31a, 19b, and 8c in Fig. 1) encoding the COOH-terminal two thirds (aa 740–1,830) of chicken brain p190 (myosin-V hc) and a portion of the flanking 3′ noncoding sequence (1,027 bp) was selected by expression screening of a chicken brain cDNA library using an antibody (Espindola et al., 1992) raised against rat brain p190. Additional cDNA clones (H11, H54, and N21; Fig. 1) covering the full-length coding region as well as a portion of flanking 5′ noncoding sequence (82 bp) were selected from the same library by two rounds of hybridization screening as detailed in Materials and Methods. The deduced amino acid sequence of chicken p190 (myosin-V hc), based on a single open reading frame of 5,572 nucleotides, encodes a 1,830–amino acid protein with a calculated unmodified molecular mass of 212,509 D (Fig. 2). This sequence was verified as encoding chicken brain p190 by amino acid sequence analysis of a CNBr fragment of the purified protein; the fragment's sequence of **X L F L K L Q K X V** exactly matched the deduced sequence between methionine 1154 and threonine 1165 (Fig. 2).

The deduced primary structure of p190 verified the suggestion from biochemical studies (Espindola et al., 1992) that this protein is a myosin. That is, like all other "myosin-like" proteins characterized thus far, chicken p190 contains an NH_2-terminal head domain similar in primary structure to that of other myosins (Figs. 3 and 4). Based on its overall primary and predicted secondary structure, p190 is most similar to the gene products encoded by the *dilute* gene of mouse (Mercer et al., 1991) and the MYO2 gene of *Saccharomyces cerevisiae* (Johnston et al., 1991; see Fig. 3 for a schematic comparison of these myosins). As detailed in the Discussion, we propose to classify the chicken p190–CM complex as a "myosin-V" based on the results of an extensive sequence comparison of the head domain of chicken brain p190 with that of other known myosins. Since the sequence of chicken brain myosin-V hc is 91% identical to the *dilute* gene product (Fig. 3) it is likely that chicken myosin-V hc is an avian homolog of the protein encoded by the *dilute* gene. As diagrammed in Fig. 3 and shown in detail in Figs. 4–7, chicken myosin-V hc and the other two members of this family of unconventional myosins are segmented into a number of discrete structural domains described in detail below.

Myosin Head Domain of Chicken Myosin-V hc. An alignment of the NH_2-terminal 764 aa of chicken myosin-V hc with the NH_2-terminal portion of other myosins revealed that this protein possesses a head domain (residues 1–764) that shares ~40% sequence identity with the head domains of most other myosins. This includes an ATP binding site identical in sequence to that of most myosins (Fig. 4). The head domain of chicken myosin-V hc is virtually identical in amino acid sequence to the head domain of mouse *dilute* (94% identity), and among other myosins it is most similar to the MYO2 gene product of yeast (52% identity). Like myosins-II, the head domain of chicken myosin-V hc is longer than that of known myosins-I because of the presence of an ~60–amino acid "extension" at its NH_2 terminus (Fig. 4). The trimethyl lysine residue found in many myosins-II (residue 125 in chicken nonmuscle myosin-II; Fig. 4) is occupied by a glutamic acid residue in the comparable position in chicken myosin-V hc. There is a single cysteine residue (position 685) within the region comparable to that containing the two cysteines found in the "active thiol" region of many myosins-II (Fig. 4; for review see Warrick and Spudich, 1987).

"Neck" Domain of Chicken Myosin-V hc. Between residues 765 and 909 (Fig. 4), there is a series of six imperfect ~23–amino acid tandem repeats, defined by a consensus sequence termed the "IQ-motif" (Fig. 5; Cheney and Mooseker, 1992) that is rich in basic residues. Five of these repeats were recognized by Mercer et al. (1991) in *dilute*; the neck domains of the unconventional myosins encoded by the MYO2 (Johnston et al., 1991) and MYO4 genes (GenBank accession No. M90057) also consist of six IQ-motifs. These repeats are similar in position and primary structure to the four to six such repeats found at the head–tail junction of BB myosin-I (Fig. 5). Several lines of evidence indicate that this is the region of BB myosin-I that is responsible for CM binding (for review see Mooseker et al., 1991; see below). The properties of the presumed CM-binding neck domain of chicken myosin-V hc and its relationship to light chain binding domains of conventional myosins are outlined in more detail in the Discussion.

Tail Domain of Chicken Myosin-V hc α-Helical Segments. The beginning of the chicken myosin-V hc (and mouse *dilute*) tail domain is defined by a segment predicted

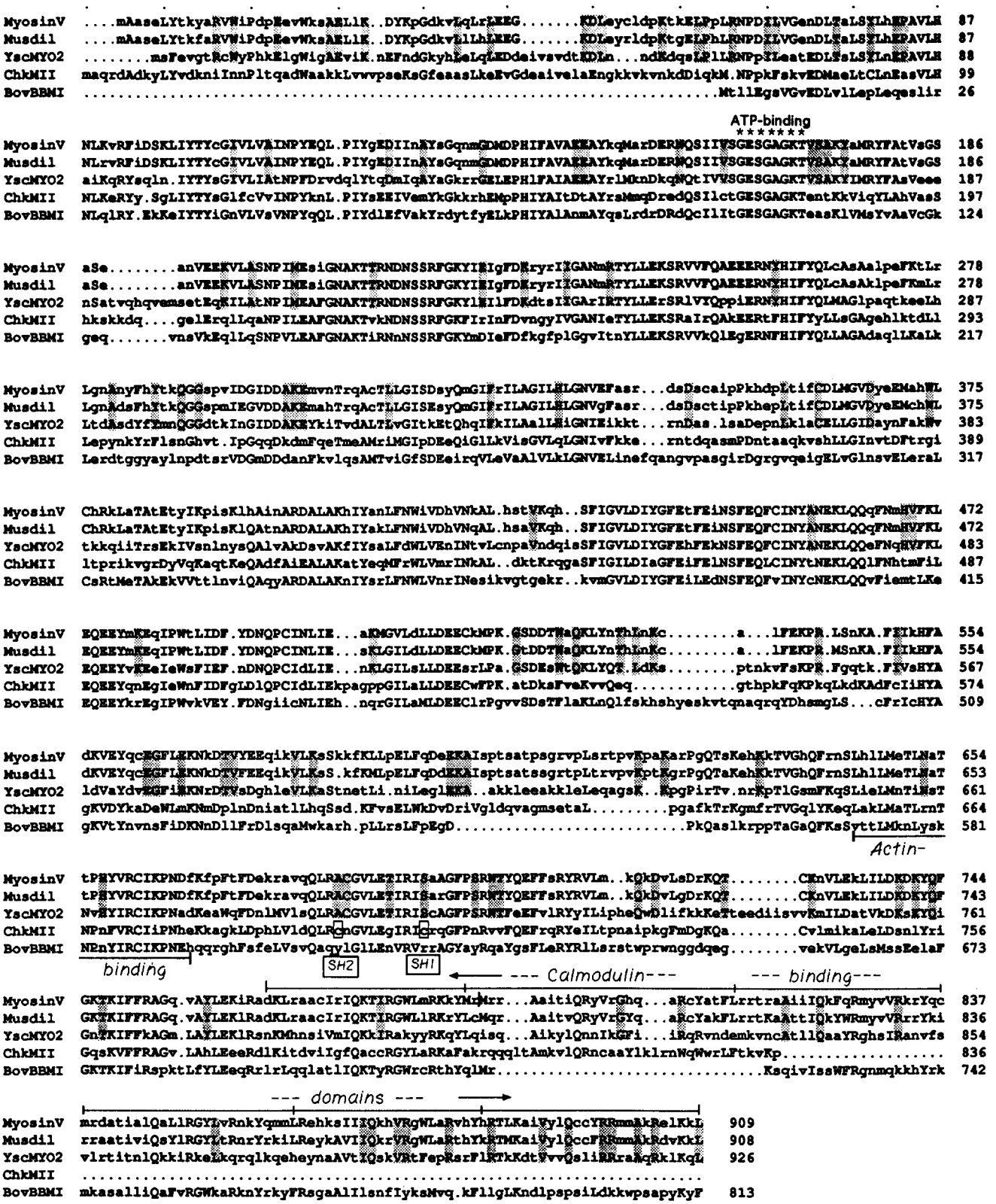


Figure 4. Comparison of the primary structure of the head and neck domains of chicken myosin-V hc (*Myosin V*) with that of conventional and unconventional myosins. Alignment of chicken myosin-V sequence with that of the mouse *dilute* gene (*Musdil*), yeast MYO2 (*YscMYO2*), a chicken nonmuscle myosin-II (*ChkMII*), and bovine brush border myosin-I (*BovBBMI*) is shown. Residues shared among chicken myosin-V hc, *dilute*, and MYO2 but not with other myosins are block-shaded. Residues in capital letters denote amino acid identity or conservative substitutions when present in at least three of the five sequences aligned. The ATP binding site, the putative actin binding site, and the calmodulin binding domains (IQ-motifs) are indicated on the chicken myosin-V sequence. The sulfhydryl-1 and sulfhydryl-2 residues found in most myosins-II are indicated by the small boxes in the chicken nonmuscle myosin-II sequence.

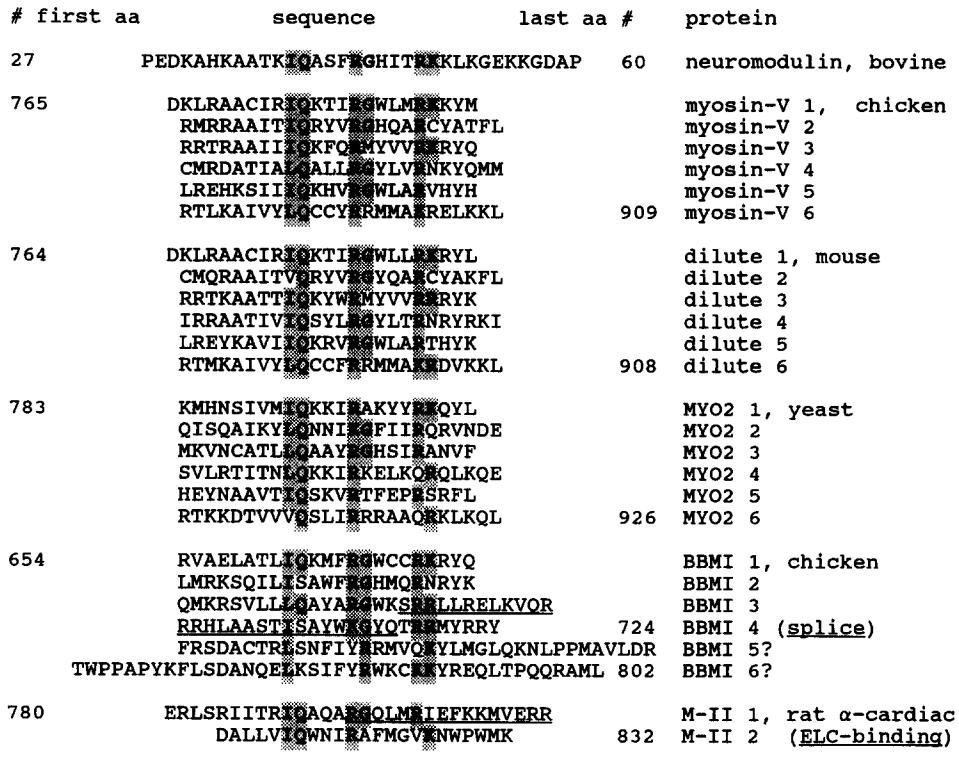


Figure 5. Alignment of the IQ-motifs from the neck domains of several myosins. These ~23-amino acid imperfect tandem repeats form putative calmodulin/light chain binding sites and are very similar to the calmodulin binding domain of the brain protein neuromodulin. Residues conforming to the consensus **IQXXXRGXXXRK** are shaded, allowing conservative I–L and R–K substitutions. The peptide corresponding to the underlined alternative splice sequence in BBMI has been shown to bind to calmodulin (Halsall and Hammer, 1990) and deletion of the underlined portion of first repeat in the rat cardiac myosin-II sequence has been shown to eliminate binding to the essential light chain (*ELC*) McNally et al., 1991).

to form a coiled-coil α helix (residues 912–1,106; Fig. 6). The tail region contains two additional segments predicted to form coiled-coil α helices (residues 1,152–1,236 and 1,322–1,420), but unlike the tail domain of conventional myosins, the remainder of the tail region (residues 1,421–1,830) is not predicted to form a coiled-coil structure (see Fig. 7 for a schematic comparison of the predicted coiled-coil structure of chicken myosin-V hc with other myosins). The three α-helical segments contain a total of 365 residues that are predicted to have a 90% or higher probability of forming coiled-coils using the program of Lupas et al. (1991). If the segments containing these 365 residues were to actually dimerize, a coiled-coil rod ~54 nm in length could be formed. Each of the three coiled-coil segments is interrupted by a single break or "hinge" of from one to nine amino acids predicted to have <90% probability of forming a coiled-coil structure. Interestingly, the 45-residue region between the first two coiled-coil domains contains a "PEST" sequence (residues 1,119–1,139; Fig. 6). PEST sequences are frequently associated with cleavage by the calcium-dependent protease calpain (Rechsteiner, 1990) and native myosin-V purified from rat brain is in fact selectively cleaved by calpain relative to conventional brain myosin (Espindola et al., 1992). The α-helical tail domain of chicken myosin-V is quite similar (87% identity) to that of mouse *dilute*, except that *dilute* contains an additional 25 amino acids inserted after residue 1386. The overall secondary structure of chicken myosin-V hc is also similar to that of MYO2, but the length of the MYO2 tail is shorter, containing only 142 residues of predicted coiled-coil (Figs. 3 and 6).

Globular, COOH-Terminal Domain of Chicken Myosin-V hc Tail. As noted above, the final 410 residues of chicken myosin-V hc are not predicted to have a highly α-helical or coiled-coil structure (Figs. 3, 6, and 7), and thus may have a globular structure. This 47-kD region of chicken myosin-V hc is remarkably conserved compared with the mouse *dilute* protein (98% amino acid sequence identity). The overall similarity of chicken myosin-V hc/*dilute* to the corresponding globular region of the MYO2 tail domain is much lower (28%), but there are regions within this domain, such as residues, 1,721–1,838, that exhibit higher local similarity. Surprisingly, a database search revealed that the globular tail domain of chicken myosin-V hc, together with a portion of its α-helical region, shares 58% sequence identity (Fig. 6) with the entire 723-amino acid–deduced sequence of a mouse protein reported to be a glutamic acid decarboxylase (Huang et al., 1990). The significance of this finding is discussed below.

Mapping the CM-binding Domain of Chicken Myosin-V hc

To directly verify that the neck domain of chicken myosin-V hc was the region of this protein involved in CM binding, sequence segments encoding the head, neck, and tail domains were expressed as bacterial fusion proteins to determine which of these domains exhibits CM binding activity. As detailed in Materials and Methods, all three domains were subcloned, in frame, into the pIH902 MBP fusion protein vector. The head construct contained chicken brain myosin-V hc residues 5–752. The neck construct contained aa 760–922, which spans the entire neck region defined above. The tail construct contained residues 899 to 1,830 and thus included, at its NH_2-terminal end, 11 aa of the sixth IQ-motif. The resulting fusion proteins exhibited electrophoretic mobilities on SDS-PAGE consistent with their predicted size (Fig. 8 *a*). CM binding to fusion proteins containing the head, neck, and tail domains was assessed by an ^{125}I-CM gel overlay

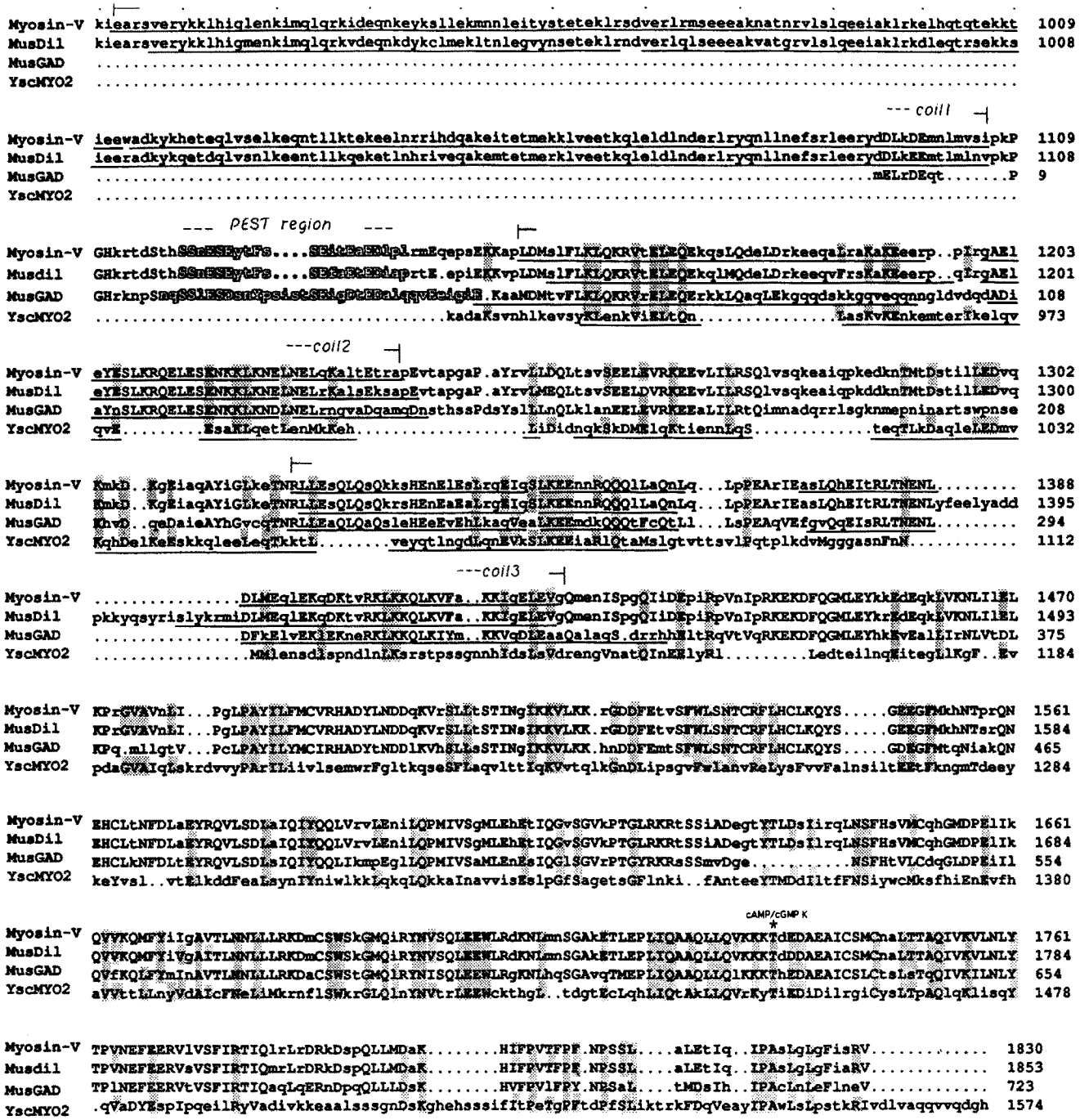


Figure 6. Comparison of the primary structure of the tail domain of chicken myosin-V hc (*Myosin*-V) with the tail domains of *dilute* and MYO2 and the deduced sequence of a mouse brain protein reported to be a glutamic acid decarboxylase (*MusGAD*). Open letters denote residues that correspond to "PEST" sequences, which are frequently associated with cleavage by the calcium-dependent protease calpain. A threonine (residue 735 in chicken myosin-V) within a consensus phosphorylation site for cAMP and cGMP kinases is indicated. Residues predicted to have >90% probability of forming a coiled-coil α helix are underlined. Capital letters indicate conservative replacements or identical residues shared among at least three of the four sequences aligned. The shaded blocks indicate residues shared by MYO2 and at least one of the other sequences.

technique (Carlin et al., 1981). Purified chick brain myosin-V together with bacterial lysates containing the induced head, neck, and tail fusion proteins were separated by SDS-PAGE, (Fig. 8 *a*) and the gels were incubated with ^{125}I-CM in the presence (Fig. 8 *b*) or absence (Fig. 8 *c*) of Ca^{2+}. The fusion protein containing the neck domain of chicken myosin-V hc exhibited prominent CM binding activity (lanes *4*, Fig. 8, *b* and *c*). No detectable binding was observed for the head domain fusion protein (lanes *3*, Fig. 8, *b* and *c*). The tail fusion protein also failed to exhibit prominent calmodulin binding (lane *5* in Fig. 8 *b*), although upon much longer autoradiographic exposures a weak signal was detected. Although this weak signal could be due to nonspecific binding, it is interesting to note that the tail construct did contain the

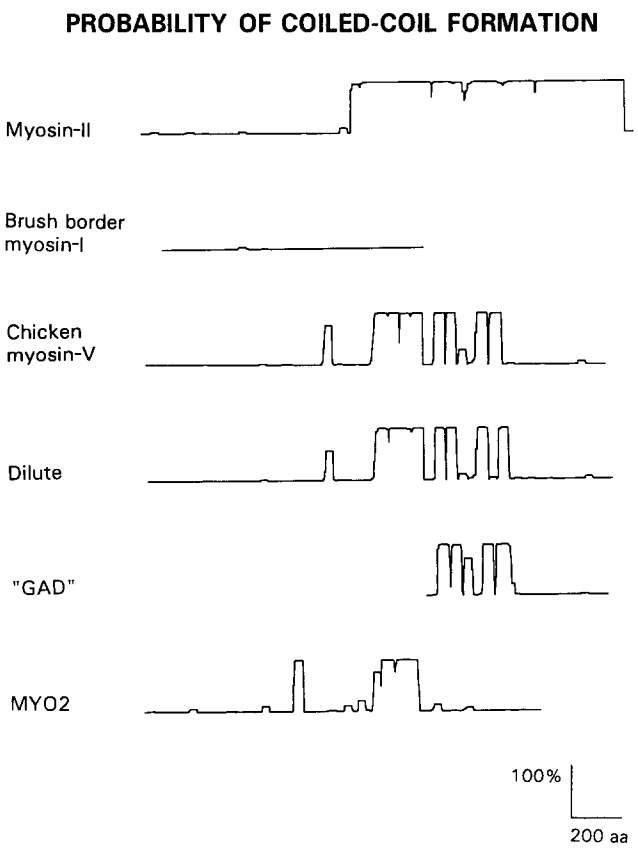


Figure 7. Schematic representation of the predicted coiled-coil secondary structure for chicken brain myosin-V hc and several other myosins. The predicted structure for the mouse protein reported to be a GAD is also shown for comparison. The height of the trace is proportional to the probability of forming a coiled-coil α helix using the coiled-coil prediction program of Lupas et al. (1991). The maximum heights shown represent 100% predicted probability.

last 11 amino acids of the final IQ-motif of the neck domain. The Ca^{2+} dependence of CM binding to the neck domain fusion protein is comparable to that of authentic chicken myosin-V (lanes *2*, Fig. 8, *b* and *c*) and chicken BB myosin-I (Lanes *1*, Fig. 8) when assayed by such overlay techniques (e.g., see Howe et al., 1982) in that optimal binding was observed in the presence of Ca^{2+}. Nevertheless, substantial CM binding to the neck domain (as well as to the hc of tissue-derived myosin-V and BB myosin-I) was observed in the absence of Ca^{2+} (Fig. 8 *c*). This is in contrast to the stringently Ca^{2+}-dependent CM binding observed with the α subunit of avian BB spectrin (lanes *1*, Fig. 8, *b* and *c*).

Localization of Myosin-V

Antibodies were raised against bacterially expressed protein containing most of the tail domain of chicken myosin-V hc (clone 32a in Fig. 1). Affinity-purified antibodies reacted specifically with a protein of an apparent molecular mass of 190 kD (Fig. 9) in whole homogenates of chick or rat brain and with the heavy chain of purified chick brain myosin-V. The very faint bands visible in the Coomassie blue–stained lanes of purified myosin-V, migrating about halfway down the gel, are due to contaminants (keratins?) present in the electrophoresis system since they were present at the same abundance in both the 1× and 0.2× dilutions of myosin-V. Like the original anti–rat brain p190 antibody (Larson et al., 1990; Espindola et al., 1992) this antibody did not cross-react with the myosin-II heavy chain(s) present in these brain homogenates or in preparations of isolated chicken intestinal brush borders (results not shown).

Using these antibodies, the localization of myosin-V in frozen sections of rat brain cerebellum and brain stem was determined (Fig. 10). Myosin-V immunoreactivity was present in all regions of the neuropil and was present at particularly high concentrations in cell bodies and dendrites of Purkinje cells (Fig. 10 *a*). Double staining of neurons with anti–myosin-V and the lectin, WGA, was performed on sections of rat brain stem (Fig. 10, *b* and *c*). Colocalization of myosin-V with WGA was observed with perinuclear cytoplasm of neuronal cell bodies, suggesting an association of myosin-V with Golgi-derived cytoplasmic membranes. In addition, immunoreactivity outlined the profile of the neuronal surface.

The distribution of myosin-V in neurons present in primary cultures derived from rat hippocampus was also examined. For these studies the anti–chicken myosin-V antibodies as well as the original anti–rat myosin-V antibodies were used; both antibodies yielded similar results (not shown). In neurons, myosin-V staining was most intense within the perinuclear region of the cell body, where it displayed a highly punctate distribution. In most cases, the cell body staining was so intense that photographic documentation of its punctate nature was difficult, although it was quite obvious by eye when focusing through the cell body. Punctate myosin-V staining was also observed along the length of dendritic and axonal processes (Fig. 11, *a* and *b*). Colocalization studies using phalloidin revealed that myosin-V colocalized with F-actin at the tips of these processes (Fig. 11 *b*). However, F-actin did not colocalize with myosin-V in the perinuclear region. An analogous pattern of myosin-V distribution was observed in the nonneuronal cells of these cultures (Fig. 12). In these cells, which are much flatter than the neurons, the intense punctate staining of the perinuclear region was particularly striking. There was also staining of the distal portions of cell processes (Fig. 12). In both neuronal and nonneuronal cells, by focusing through the cell body it was clear that the nucleus itself was not stained and that much of the perinuclear staining was intracellular.

Discussion

The preliminary biochemical analyses of p190 suggested that this unusual CM binding protein from brain may be a myosin—albeit with some unusual properties such as the lack of the K-EDTA ATPase activity (Larson et al., 1990; Espindola et al., 1992) that is a hallmark of most myosins. The determination of the primary structure of chicken p190 has confirmed both suggestions; this protein is indeed a myosin—and an odd one at that for which we propose the name myosin-V (see below for our rationale). An unexpected windfall from the task of determining the sequence of chicken myosin-V hc is that this protein is a homolog of the product encoded by the mouse *dilute* gene (Mercer et al., 1991) and is structurally similar to the yeast MYO2 gene product. We use the term "a" rather than "the" homolog of the mouse *dilute* gene product because the chicken myosin-V hc sequence aligns almost exactly with that of *dilute* except

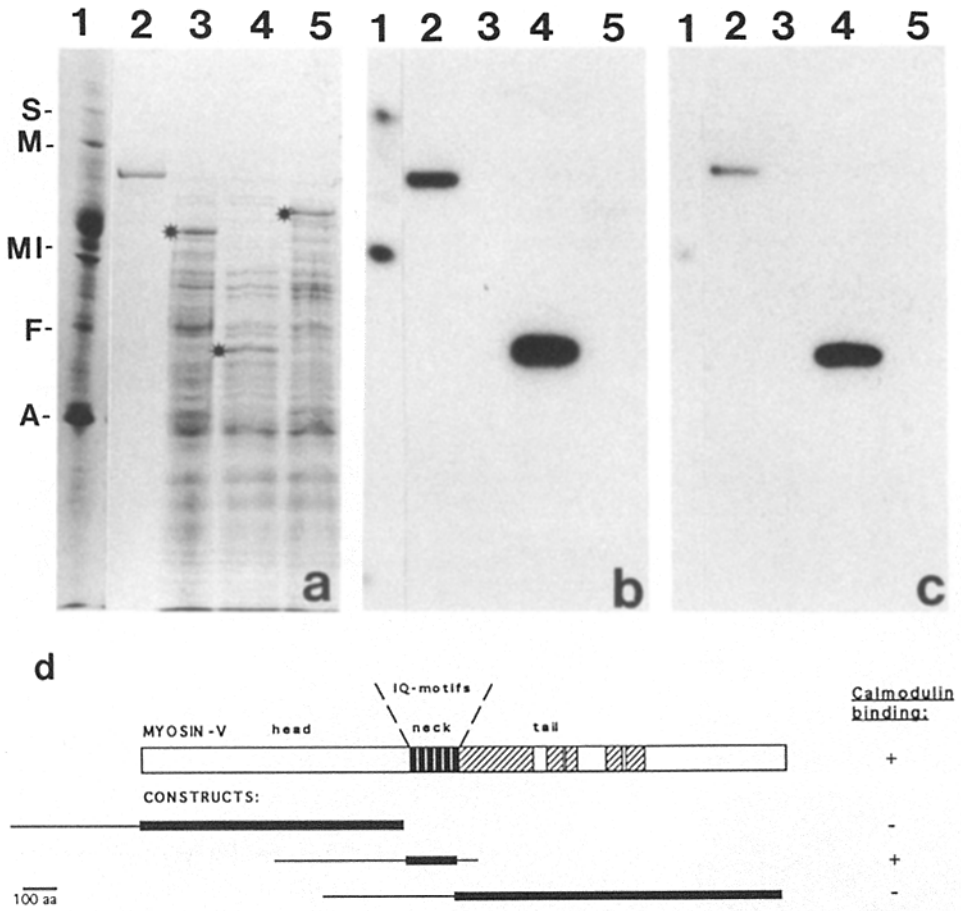


Figure 8. Identification of the neck domain as the calmodulin binding domain of chicken myosin-V hc. Coomassie-stained SDS gel (*a*) and autoradiographs (*b* and *c*) of gels incubated with ^{125}I-calmodulin either in the presence (*b*) or absence (*c*) of Ca^{2+}. Protein samples include isolated chicken intestinal BBs (lanes *1*), purified chick brain myosin-V (lanes *2*), and bacterial lysates from cells expressing fusion protein constructs containing the head (lanes *3*), neck (lanes *4*), or tail (lanes *5*) domains of chicken myosin-V hc. The letters at left correspond to the migration position of major proteins of the BB cytoskeleton including the α-subunit of BB spectrin (*S*; 240 kD), myosin-II hc (*M*; 200 kD), BB myosin-I hc (*MI*; 110 kD), fimbrin (*F*; 68 kD), and actin (*A*; 43 kD). The asterisks denote the Coomassie-stained bands corresponding to the head, neck, and tail fusion proteins. (*d*) Schematic of the fusion protein constructs containing the head, neck, or tail domains of myosin-V hc. Protein encoded by vector sequence is denoted by the thin lines.

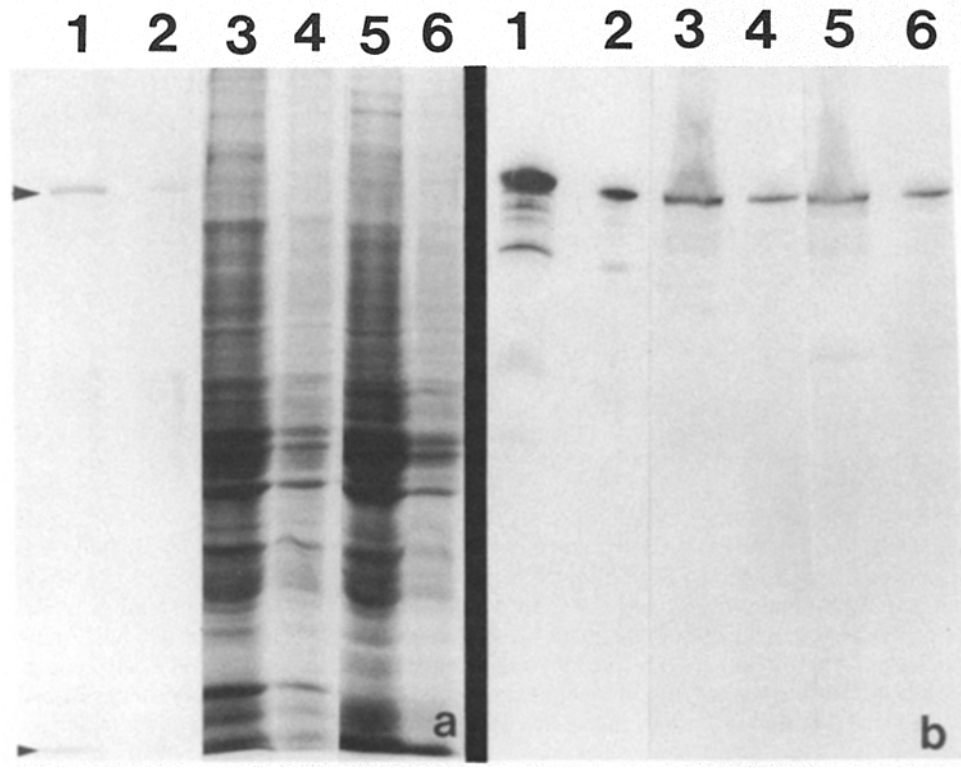


Figure 9. Immunoblot characterization of antibodies raised against bacterially expressed protein tail domain of chicken myosin-V hc. Coomassie blue-stained gel lanes (*a*) and immunoblots from an identically loaded gel (*b*) containing two different dilutions each of purified chick brain myosin-V (lanes *1* and *2*), and SDS extracts from chick (lanes *3* and *4*) and rat (lanes *5* and *6*) brain. The arrowheads indicate the position of the myosin-V hc and CM light chain bands. Lane *1* was loaded with ~1 μg of purified myosin-V, whereas lanes *3* and *5* were each loaded with ~0.1 mg of total protein; approximately one-fifth as much protein was loaded in lanes *2*, *4*, and *6*. The additional bands below 190 kD stained by anti-myosin-V in lane *1* appear to be breakdown products present in the purified myosin-V sample since their staining intensity increases with storage time.

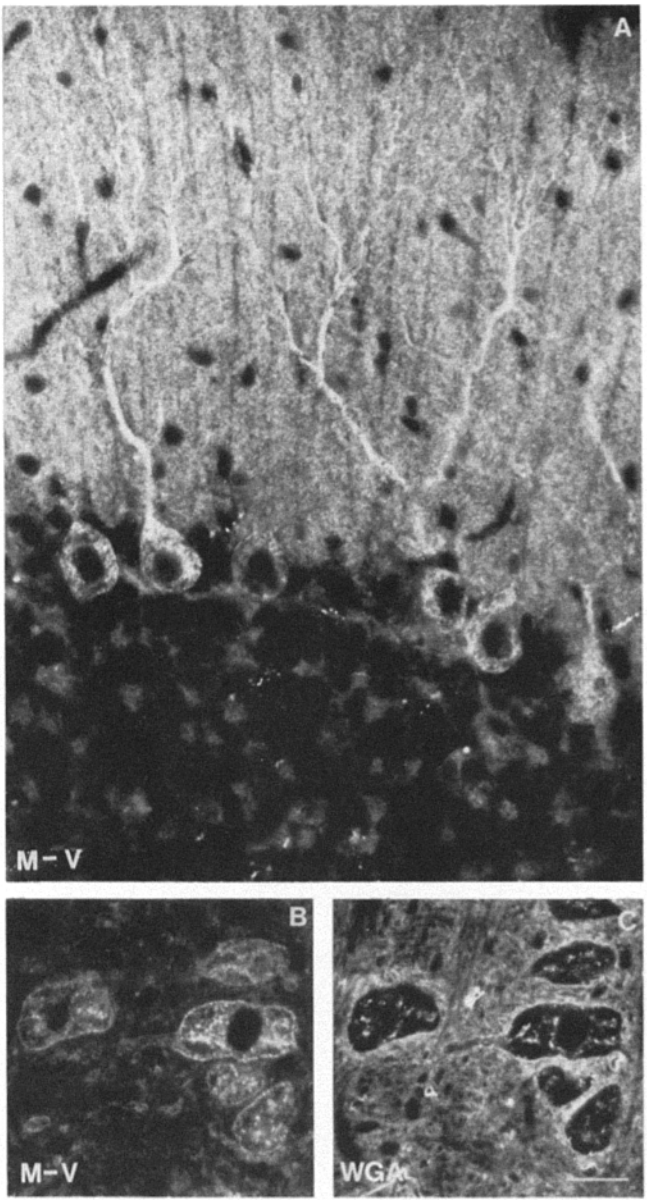


Figure 10. Immunolocalization of myosin-V heavy chain (*M-V*) in tissue sections of rat cerebellum (*A*) and brain stem (*B*) using anti-chicken brain myosin-V antibodies. Prominent staining of Purkinje cell bodies and dendrites is observed (*A*). Myosin-V immunostaining of cytoplasmic organelles within neuronal cell bodies in brain stem (*B*) colocalizes with intracellular membranes stained by the lectin WGA (*C*). Bar, 20 μm.

for a 25-aa segment found in the tail domain of *dilute* but not in chicken myosin-V hc. A likely explanation for this segment is that it is the result of alternative RNA splicing; thus it is possible that additional isoforms of myosin-V may be expressed. Previous Northern analysis has indicated that the pattern of *dilute* transcription is complex, with transcripts of 7, 8, and 12 kb present in most tissues (Mercer et al., 1991); we have detected hybridization to a 10-kb transcript in chicken. In addition to confirming that p190 is a myosin, the analysis of its primary structure has provided a number of important insights, discussed in detail below, with respect to its domain organization and relationship to other myosins.

p190-CM Complex, dilute, *and MYO2 Gene Products Are Members of a Structurally and Evolutionarily Distinct Class of Unconventional Myosins—The Myosins-V*

p190, *dilute*, and MYO2 clearly form a novel class of myosins that are distinguished by four features: (1) myosin-like head domains that are more similar to one another than to those of other myosins, (2) a neck domain that contains six tandem repeats of the calmodulin/light chain binding IQ-motif, (3) a proximal tail domain consisting of segments predicted to form coiled-coil α helices, and (4) a probably globular distal tail domain of ~45 kD of unknown function. It is obvious, based on this predicted domain structure that these myosins cannot be readily assigned to either the myosin-I or the myosin-II class. Although these myosins are likely to be two headed (based on the segments of predicted coiled-coil), they clearly have other features, such as the presence of an ~47 kD presumably globular tail domain, that make them distinct from the conventional myosins-II.

To assess whether or not we could establish the evolutionary/structural relationship of p190 (chicken myosin-V) to other known myosins, we have conducted an extensive amino acid sequence comparison among the head domains of myosins using a distance matrix and the neighbor-joining method of Saitou and Nei (1987). In the rootless phylogenetic tree generated by this procedure, the distance along the branches connecting two sequences is a measure of the percent difference in their amino acid sequence. A rootless tree generated from the amino acid sequences of 29 myosin head domains shows that p190, along with MYO2 and *dilute*, do indeed fall into a discrete class of myosins distinct from the myosins-I or the myosins-II (Fig. 13). Based on its head sequence, the newly identified unconventional myosin from yeast, MYO4 (Genbank accession No. M90057), also appears to be a member of this class. It is interesting to note that this tree of head domain sequences also indicates that *Drosophila* ninaC (Montell and Rubin, 1988; class III), the *Acanthamoeba* high molecular weight "myosin-I" (we would suggest terming this a class IV myosin), and the *Drosophila* 95F unconventional myosin (Kellerman and Miller, 1992; class VI), each constitute a separate class of myosin. The term myosin-V posed for the p190–CM complex is simply based on the chronologic order in which this class of unconventional myosins was described. It is important to note that the precise order of origin and divergence among the major myosin lineages is uncertain, as indicated both by the very short branch lengths that join the major myosin lineages at the center of the tree and by lower bootstrapping values for these nodes. Nevertheless, the division of the known myosins into six major families appears to be a robust result (a more detailed discussion of this phylogenetic tree analysis will be published elsewhere).

Neck Domain of Chicken Myosin-V hc Is Comprised of Six Tandem IQ-Motifs; These Motifs Provide Binding Sites for Calmodulin/ Light Chains

One of the striking findings arising from the sequencing of chicken myosin-V hc is the recognition that its neck domain consists of six imperfect tandem repeats of motif whose most

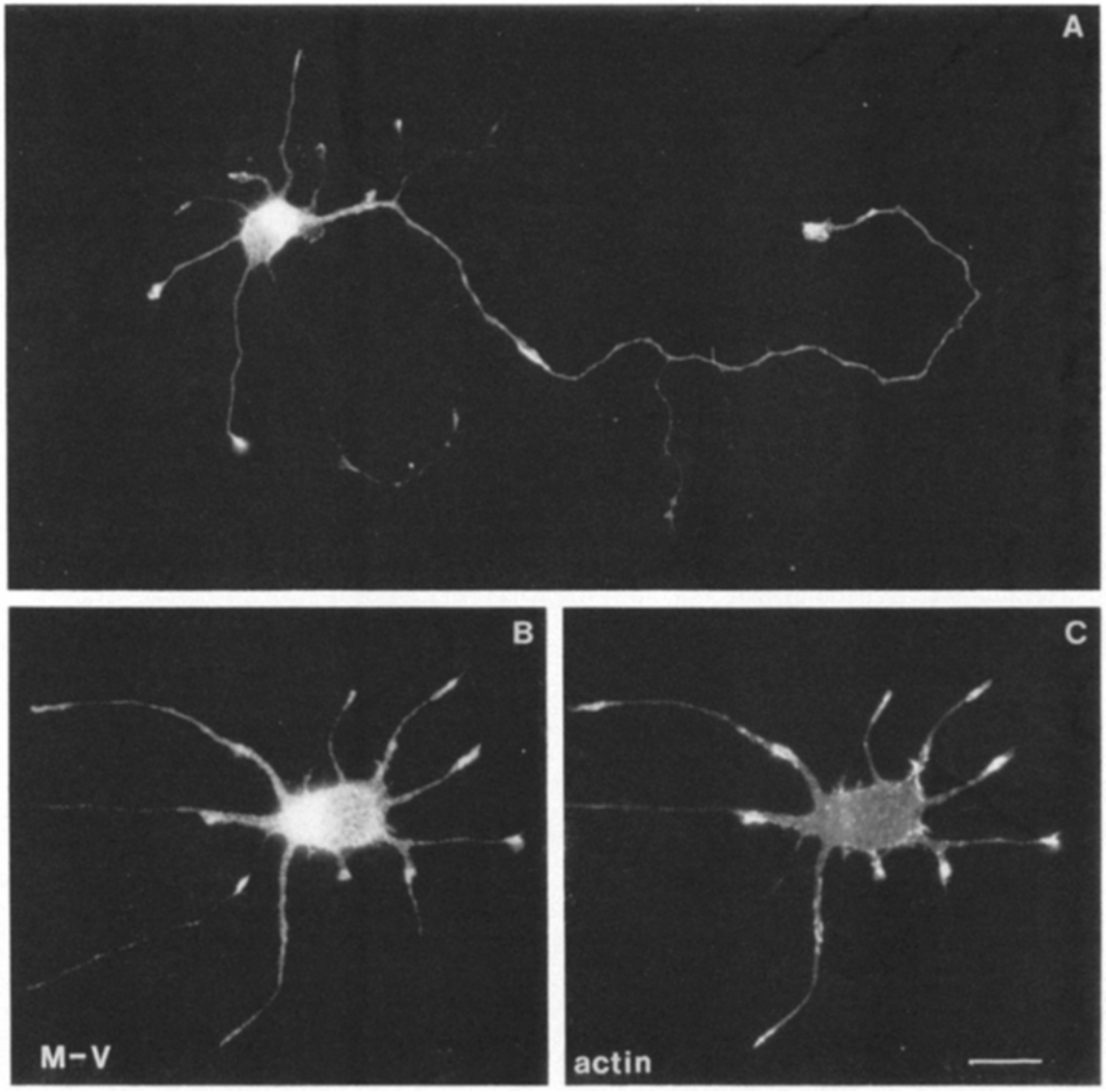


Figure 11. (*A*) Immunolocalization of myosin-V (*M-V*) in a neuron in primary culture (3-day) with an axonal process using anti-chicken brain myosin-V antibodies. Note prominent cell body and growth cone staining. (*B* and *C*) Colocalization of myosin-V (*B*) and F-actin (*C*) in a cultured neuron. Note coincidence of staining at tips of neurite processes. Bar, 12 μm.

conserved core residues can usually be represented as IQXXXRGXXXR. Each repeat is ~23 aa long, and as can be seen in Fig. 5, positions in addition to those shown above exhibit some conservation. Acidic residues and proline are rare in the central portion of the repeat, and most of the remaining residues are either basic or hydrophobic. The first five repeats in chicken brain myosin-V share an average of 38% amino acid sequence identity with one another. Although the sixth repeat shares less sequence identity, it does retain the core residues of the IQ-motif. The first IQ-motif in chicken myosin-V shares 42% sequence identity with the intensively studied (Chapman et al., 1991) CM binding domain of the neuronal protein neuromodulin, which suggested that the IQ-motifs might function as CM binding sites. Dilute (Mercer et al., 1991), MYO2 (Johnston et al., 1991), and MYO4 also contain six such motifs, although unlike brain myosin-V, which is known to have CM light chains (Espindola et al., 1992), these genes have not been studied as proteins and thus their light chain composition is currently unknown. Purified brain myosin-V is similar to chicken BB myosin-I in that both myosins contain multiple CM light chains, although the exact number of CMs associated with the heavy chain of brain myosin-V is not yet known and there is disagreement about whether chicken BB myosin-I is isolated with three or four CM light chains (Collins et al., 1990; Hayden et al., 1990; Swanljung-Collins and Collins, 1991; for review and discussion see Mooseker et al., 1991). Inspection of the chicken BB myosin-I sequence has revealed that the neck region of this myosin, which has been previously implicated as the site of CM binding (Coluccio and Bretscher, 1988; Carboni et al., 1988; Garcia et al., 1989; Halsall and Hammer, 1990; Hayden et al., 1990), contains at least three and possibly two other poorly conserved IQ-motifs (Mercer et al., 1991; Cheney and Mooseker, 1992; Swanljung-Collins and Collins, 1992). The alternative splice insert identified by Halsall and Hammer (1990) would lead to the insertion of an additional IQ-motif in BB myosin-I, and a peptide corresponding to this 29-amino acid insert was

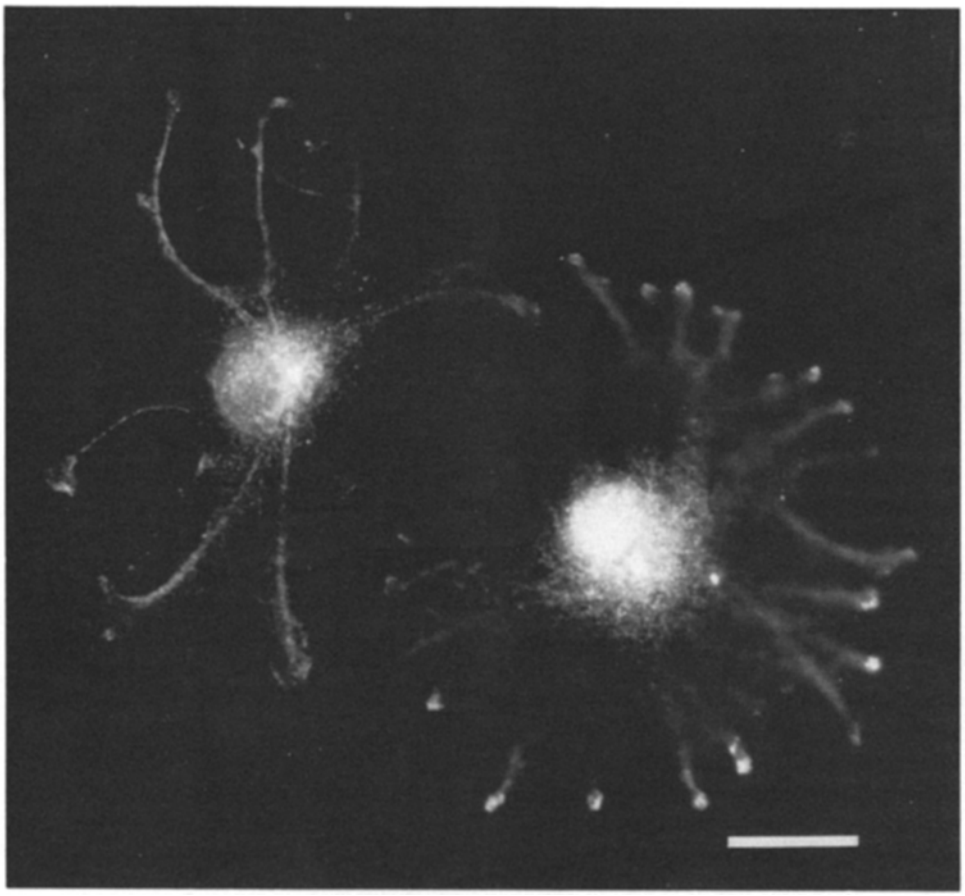


Figure 12. Immunolocalization of myosin-V (using anti-rat p190 antibodies) in astrocytes present in primary cultures of rat hippocampal tissue. Bar, 20 μm.

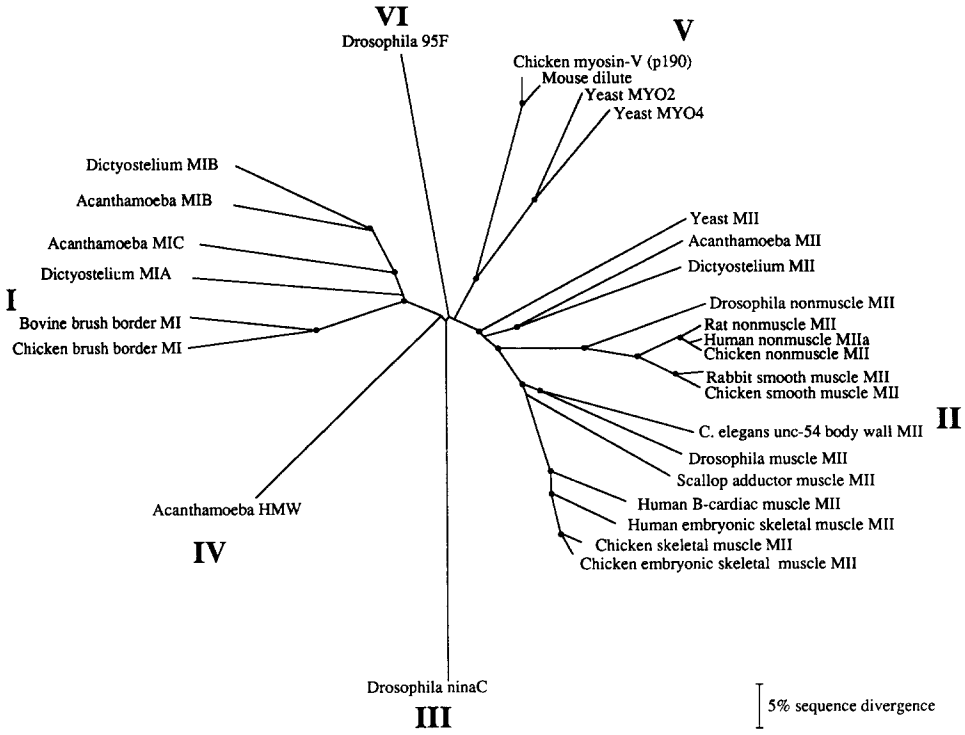


Figure 13. An unrooted phylogenetic tree of the myosin head domains. The distance along a given branch or branches connecting two sequences is proportional to the percent amino acid sequence difference between the two sequences. Sequences and sets of sequences that were joined together in at least 980 out of 1,000 data resampling (bootstrapping) trials are indicated by the nodes marked with small filled circles. Note that the known myosins clearly fall into six distinct families (indicated by roman numerals) based on their head sequences but that the branching order at the "center" of the tree is uncertain. Note also that chicken myosin-V, *dilute*, MYO2, and the newly identified MYO4 constitute a group distinct from the myosins-I or the myosins-II. The alignment and conversion of these sequences into a tree was performed using the Clustal V program.

shown to bind to CM. Although previous studies of proteolytically cleaved chicken BB myosin-I indicate that its CM binding sites are near the head–tail junction (Coluccio and Bretscher, 1988; Carboni et al., 1988), the precise boundaries of this region are not known. By producing three constructs that encoded either the head, the neck, or the tail domain of chicken myosin-V hc, we were able to demonstrate that the neck domain contains the CM binding sites detectable by ^{125}I-CM overlay. Because this is the region that consists of the six IQ repeats, this work demonstrates that the CM binding sites of chicken myosin-V hc map precisely to the region of the IQ-motifs. Whether all six of these motifs are actually functional under native conditions, and what their affinities for CM are, remains unknown. In addition, we note that chicken myosin-V hc, chicken BB myosin-I hc, and neuromodulin all have the unusual property of binding to CM in the absence of calcium. This suggests that CM binding sites formed by the IQ-motifs may differ from the more conventional calcium-dependent CM binding sites.

It is important to note that all myosins of known primary structure contain one or more sequences analogous to the IQ-motif in their neck domains (Mercer et al., 1991; Cheney and Mooseker, 1992). The myosins-II contain two such repeats, although the second repeat is poorly conserved in its latter half and it ends just ahead of the proline that defines the beginning of the myosin-II tail domain. Numerous studies (Mitchell et al., 1989; Nyitray et al., 1991) have implicated the ~100 amino acids in the myosin-II "neck" domain as providing the primary binding sites for the essential and regulatory myosin light chains. Moreover, a recent study has demonstrated that deletion of 16 amino acids from what we would define as the first IQ repeat in rat cardiac myosin eliminates binding to the essential myosin light chain (McNally et al., 1991). Since both of the myosin light chains are members of the calmodulin/EF-hand superfamily of proteins (Kretsinger, 1980), we hypothesize that IQ-motifs provide binding sites for calmodulin or for related proteins of the EF-hand superfamily. This raises the possibility that all myosins share a similar structural basis for regulation by their light chains.

Is the Tail of Myosin-V a Glutamic Acid Decarboxylase (GAD)?

As noted in Fig. 6, the tail domain of chicken myosin-V (and *dilute*) is highly similar to the entire deduced structure of an ~80-kD mouse protein reported to be a GAD (Huang et al., 1990). This type of enzyme is responsible for the decarboxylation of glutamic acid to yield the neurotransmitter GABA. Beginning at residue 1,093 in the tail domain of chicken myosin-V hc, the putative mouse GAD shares 58% sequence identity with the remaining 737 amino acids of the tail domain. The GAD sequence includes 185 residues predicted to form a coiled-coil as well as a PEST sequence. It is important to note that the mouse GAD and the tail domain of mouse *dilute* are not identical in sequence (57% sequence identity). Thus, the *dilute* gene product and the GAD described by Huang et al. (1987) are not products of the same gene through alternative splicing. The implications of the great similarity between the tail domain of myosin-V and a neurotransmitter synthesizing enzyme are potentially quite striking. However, several lines of evidence makes us view this finding with skepticism. First, the GAD sequence described by Huang et al. (1990) lacks a consensus binding site for the GAD cofactor pyridoxal phosphate and shows no sequence similarity to other well-characterized GAD enzymes (Kobayashi et al., 1987; Erlander et al., 1991; Karlsen et al., 1991). Second, in preliminary studies we have been unable to detect GAD activity associated with a highly purified preparation of native myosin-V from chicken brain (our unpublished observation); however, such a negative result could obviously be due to an inactivation of the GAD activity during purification. Third, in the report by Huang et al. (1990), in which bacterially expressed GAD was reported to have enzymatic activity, GAD activity was measured only in a crude bacterial lysate and thus it is not clear what the specific activity of the cloned protein actually is. Given these uncertainties it is reasonable to consider other alternative explanations. First, given the lack of similarity in primary structure to several other GADs, it is possible that the cDNA sequence described by Huang et al. (1990) encodes a protein that is neither a GAD nor a myosin. This possibility would suggest that a protein very similar in structure to the tail domain of myosin-V exists as a separate functional unit; we note that proteolytic cleavage of myosin-V hc near its PEST sequence would produce a very similar unit. Another possibility is that Huang et al. (1990) actually cloned a partial cDNA (with an artifactual initiation codon at the 5′ end) encoding the tail domain of another member of the class V family of myosins. Given the existence of both MYO2 and MYO4 in yeast, and the existence of additional mouse genes with mutant phenotypes similar to those of *dilute* (Silvers, 1979; Moore et al., 1990), it would not be surprising if vertebrate brain contains additional members of the myosin-V family of unconventional myosins. This possibility that the putative GAD represents part of an ~200-kD unconventional myosin instead of an ~80-kD GAD cannot be ruled out on the basis of transcript size since Huang et al. (1990) did not report any Northern blotting results. It will be prudent, with respect to dissecting the function of the class V family of myosins, to determine which, if any, of the above possibilities is correct.

Is the Myosin-V Family of Myosins Involved in Organelle Transport?

The immunolocalization studies presented here, in combination with the characterization of mutants for the *dilute* and MYO2 genes, provide a firm basis for posing focused questions regarding the functions for this structural class of myosins. Although immunochemical studies (Espindola et al., 1992) indicate that myosin-V is expressed in a wide range of tissues, it exhibits highest levels of expression, based on immunoblot analysis, in brain tissue. The immunolocalization studies reported here show that myosin-V is expressed in both neurons and nonneuronal cells of the brain. In neurons, myosin-V exhibits an interesting pattern of localization that is seen in neurons both in situ (Fig. 10) and in primary culture (Fig. 11). High levels of a punctate and perinuclear immunostaining are observed in both neurons and glial cells. We demonstrate that this is likely to be in part due to staining of the Golgi region, since the p190 colocalizes with perinuclear cytoplasmic organelles reactive with the lectin WGA (Fig. 10, *b* and *c*). A punctate staining pattern is also ob-

served for myosin-V along the lengths of cell processes; this staining is particularly bright at the distal ends of cell processes. The surface staining that was also observed on neurons argues that some myosin-V is associated with either the membrane-associated cytoskeleton or with the plasma membrane. An obvious speculation from the localization data presented here is that myosin-V associates with Golgi-derived cytoplasmic vesicles that are eventually transported out to the cell periphery. Myosin-V could play an active role in this transport process. Alternatively, it could merely be a passenger on vesicles that are transported along microtubules until they reach the cell periphery. Once delivered there, myosin-V might function by interacting with the actin-based cytoskeleton either before or after a vesicle's fusion with the plasma membrane. A possible morphological correlate of such roles for myosin-V has been reported by Kuznetsov et al. (1992), who observed that vesicles from extruded squid axoplasm initially moving along microtubules sometimes switch over to move along actin filaments.

The phenotypes described for mutant alleles of the *dilute* gene are compatible with either of the two general roles outlined above. For example, the original *dilute* mutation is apparently restricted in its effects to melanocytes, where no product is expressed (Silvers, 1979). In these mice, the melanocytes fail to form dendritic processes and melanosomes are not effectively transferred to hair shaft keratinocytes, resulting in a "dilution" of coat color (Silvers, 1979). As discussed by Mercer et al. (1991), the *dilute* gene product may be involved in melanosome transport, or alternatively, it may be required for some polarization process involved in dendrite formation. In addition to such tissue-specific *dilute* mutations, there are *dilute lethal* alleles, including deletion mutants, that cause severe neurological defects such as seizures. Interestingly, although such mutations are lethal, the animals generally live for a few weeks after birth and at the gross anatomical level exhibit no major defects in their nervous system (Silvers, 1979). Thus, the *dilute* gene product is clearly not required for developmental phenomena such as extension of axons and dendrites. Given these findings, it seems unlikely that class V myosins are involved in transport of a population of vesicles that are essential for growth and extension of axons and dendrites. Rather, it seems more likely that this class of myosins is involved in differentiation-specific functions such as neuronal transmission. Because our studies have shown that myosin-V immunogens are not restricted in expression to neuronal cell types, it may be quite informative to examine what effect, if any, such mutations have on the function and cytological organization in nonneuronal cells in which this myosin is expressed.

Substantive insights regarding the functional properties of the class V family of unconventional myosins are also provided by studies on the MYO2 gene by Johnston et al. (1991). At the nonpermissive temperature, the temperature-sensitive myo2-66 mutants form large cells which fail to develop buds. The actin cytoskeleton is disrupted in these cells and the cytoplasm contains abnormally large numbers of cytoplasmic vesicles. Obviously, such a build-up in cytoplasmic vesicles could result from defective transport to the plasma membrane or, alternatively, a failure of those vesicles to appropriately dock and fuse with the plasma membrane at the bud. On the other hand, the disruption of the actin cytoskeleton also observed in these mutant cells could be the primary defect that leads to a secondary disruption of vesicle transport or targeting. It is interesting to note, however, that preliminary evidence indicates that the myo2 mutation exhibits the genetic interaction known as synthetic lethality with the late acting (post-Golgi) *sec* mutants but not with the *sec* mutants acting earlier in the secretory pathway (Govindan, B., R. Bowser, and P. Novick, unpublished results). Consideration of MYO2's function has become an even more fascinating exercise in light of recent studies by Lillie and Brown (1992) in which a multicopy suppressor for the myo2 mutation was identified (termed SMY1) that encodes a novel protein with a "head" domain similar in primary structure to that of the microtubule-based mechanoenzyme, kinesin. These results raise the general question of the relative roles of microtubule- and actin-based motor proteins in cell function.

It is apparent that the availability of the above mutations should continue to provide powerful experimental approaches to dissecting the functions of this class V family of unconventional myosins. However, it is also clear that further studies of the biochemical and cell biological properties of the proteins encoded by these genes are essential to sort out which if any of the alternative mechanisms discussed above are operative. Some critical open questions that should be addressed include the following: Is chicken myosin-V an active mechanoenzyme and, if so, in what direction along an actin filament does it move? Are there multiple isoforms of myosin-V expressed in a given organism—in a given cell? How many CM light chains are actually associated with the heavy chain of myosin-V, and what is the oligomeric state of myosin-V under native conditions? What is the role of the multiple CM light chains in regulating its mechanochemical properties and presumed association with membranes? What is the role of myosin-V hc phosphorylation by CM-dependent kinase II (Larson et al., 1990)? With what class of cytoplasmic organelles is myosin-V associated and do those organelles also contain microtubule-based motors? Fortunately, with the availability of excellent antibody reagents for myosin-V, together with the recent development of new methods for purification of preparative amounts of this myosin from vertebrate brain (Cheney, R. E., M. K. O'Shea, M. V. Coelho, E. M. Espreafico, J. W. Wolenski, J. Heuser, P. Forscher, R. E. Larson, and M. S. Mooseker, manuscript in preparation) many of the above questions will soon be answered.

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Note Added in Proof. The p190 protein recently identified by Settleman, J., V. Narasimhan, L. C. Foster, and R. A. Weinberg (*Cell.* 1992. 69: 539–549) shares no sequence similarity with the protein reported here.

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