Identification and Characterization of Nonmuscle Myosin II-C, a New Member of the Myosin II Family*

Eliahu Golomb‡§, Xuefei Ma‡, Siddhartha S. Jana‡, Yvette A. Preston‡, Sachio Kawamoto‡, Nitza G. Shoham‡, Ehud Goldin**, Mary Anne Conti‡, James R. Sellers‡, and Robert S. Adelstein‡

From the §Laboratory of Molecular Cardiology, NHLBI, the ¶Genetics and Genomics Branch, NIAMS, and the **Developmental Metabolic Neurology Branch, NINDS, National Institutes of Health, Bethesda, Maryland 20892 and the §Department of Pathology, Shaare Zedek Medical Center, Jerusalem 91031, Israel

A previously unrecognized nonmuscle myosin II heavy chain (NMHC II), which constitutes a distinct branch of the nonmuscle/smooth muscle myosin II family, has recently been revealed in genome data bases. We characterized the biochemical properties and expression patterns of this myosin. Using nucleotide probes and affinity-purified antibodies, we found that the distribution of NMHC II-C mRNA and protein (MYH14) is widespread in human and mouse organs but is quantitatively and qualitatively distinct from NMHC II-A and II-B. In contrast to NMHC II-A and II-B, the mRNA level in human fetal tissues is substantially lower than in adult tissues. Immunofluorescence microscopy showed distinct patterns of expression for all three NMHC isoforms. NMHC II-C contains an alternatively spliced exon of 24 nucleotides in loop I at a location analogous to where a spliced exon appears in NMHC II-B and in the smooth muscle myosin heavy chain. However, unlike neuron-specific expression of the NMHC II-B insert, the NMHC II-C inserted isoform has widespread tissue distribution. Baculovirus expression of noninserted and inserted NMHC II-C heavy meromyosin (HMM II-C/HMM II-C1) resulted in significant quantities of expressed protein (mg of protein) for HMM II-C1 but not for HMM II-C. Functional characterization of HMM II-C1 by actin-activated MgATPase activity demonstrated a Vmax of 0.55 ± 0.18 s−1, which was half-maximally activated at an actin concentration of 16.5 ± 7.2 μM. HMM II-C1 translated actin filaments at a rate of 0.05 ± 0.011 μm/s in the absence of tropomyosin and at 0.072 ± 0.019 μm/s in the presence of tropomyosin in an in vitro motility assay.

Myosins are protein molecular motors that bind to filamentous actin in an ATP-dependent manner. They are expressed as numerous classes in all eukaryotic cells (1). Nonmuscle myosins include the ubiquitous conventional myosins (class II), which form filaments at relatively low ionic strength and share a number of biological properties with skeletal, cardiac and smooth muscle myosin (1). They are expressed in both muscle and nonmuscle cells and are hexamers, consisting of a pair of heavy chains (200 kDa) and two pairs of light chains (20 and 17 kDa). Nonmuscle myosin II is one of the main motors interacting with cytoskeletal actin and is involved in regulating cytokinesis, cell motility, and cell polarity in many eukaryotic cells (1, 2).

Two different nonmuscle myosin IIs have been described to date that are referred to as myosin II-A and II-B (3–9). These names are based on their unique heavy chain polypeptides, which are encoded by two different genes, located in humans on chromosomes 22 and 17 and in mice on chromosomes 15 and 11, respectively. It is presently not known whether nonmuscle myosin heavy chain (NMHC)1 II-A and II-B share the same or different light chain isoforms. There is one known form of NMHC II-A, whereas four alternatively spliced forms of NMHC II-B have been described (1, 8, 9).

The localization of NMHCs II-A and II-B has been described for a number of different cultured cells and tissues (10–14), but no consistent pattern has emerged. Depending on the cell type, there appear to be areas that contain a particular myosin II isoform, but there are also areas where they clearly overlap at the light microscopic level. Some cells appear to express only one isoform, and a number of different approaches have been utilized to lower or remove one of the isoforms from both cultured cells (15, 16) and mice (17–19). Specific interactions between nonmuscle myosin II-A or II-B isoforms with a variety of proteins have been reported. These include the interaction of nonmuscle myosin II-B with the PBX family of homeodomain transcription factors (20) and the interaction of nonmuscle myosin II-A with the tumor suppressor protein menin (21), the CXCR4 chemokine receptor (22), and the S100 protein mts1 (23).

Analysis of genomic and cDNA data bases raised the possibility of the existence of previously unrecognized myosins, including myosin IIs (24). Expressed sequence tags (ESTs) have occasionally been annotated as “moderately similar to nonmuscle myosin II-B” (or "II-A"), and a potential NMHC gene has been noted on human chromosome 19 (accession number AC020906) (24). Recently, Leal et al. (25) also found that this gene, MYH14, is transcribed into RNA and that this RNA is particularly abundant in human intestine and skeletal muscle.

The purposes of the present study were: 1) to verify that the putative gene encoding a third NMHC, which we refer to as II-C, is translated into a previously unrecognized protein; 2) to determine what differentiates this protein from other nonmuscle myosin IIs; 3) to examine whether it is expressed in different alternatively spliced forms; and 4) to biochemically characterize its properties as a myosin.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Pathology, Shaare Zedek Medical Center, Jerusalem 91031, Israel. E-mail: golomb@szmc.org.il.

‡ The abbreviations used are: NMHC, nonmuscle myosin heavy chain; EST, expressed sequence tag; MOPS, 3-[N-morpholino]propane-sulfonic acid; HMM II-C, noninserted heavy meromyosin, HMM II-C1, inserted heavy meromyosin; RT, reverse transcription; E, embryonic day n.
EXPERIMENTAL PROCEDURES

Identification of cDNA Sequence—BLAST searches of EST and genomic data bases were used to search for new nonmuscle myosins. The LEADSTM platform for clustering and assembly of DNA and cDNA sequences at (www.labonweb.com) was used to cluster and assemble ESTs and for the search of alternative splice variants. This analysis led to the identification of human and mouse RNAs and ESTs encoding a previously unrecognized nonmuscle myosin that we termed II-C.

Sequences used to identify NMHC II-C included the human genomic sequence corresponding to nucleotides 6398279–64255191 of chromosome 19 (based on a BLAT search at genome.ucsc.edu/cgi-bin/hgBlat) and to accession numbers AC020906, AC010515, and AC019157 and the mouse genomic sequences AC073782 and AC073806, as well as other ESTs and annotated RNA sequences clustered in Unigene clusters Hs.115412/Hs.250389 and Mm.7112 (mouse chromosome 7). Comparison of the human ESTs BG829206 and BG468611 indicated that there are two alternatively spliced forms of NMHC II-C, the longer of which contains a cassette insert of 24 nucleotides derived from an exonic exon. Electronic gene expression data were retrieved from the websites www.ncbi.nlm.nih.gov/SAGE and bodymap ims.u-tokyo.ac.jp.

RNA isolation and RT-PCR—Total RNA was extracted from mouse tissues using Trizol Be Reagent (Tel-Test Inc., Friendswood, TX). Poly(A) RNA was isolated from total RNA using the NucleoTrap mRNA purification kit (BD Clontech, Palo Alto, CA). Reverse transcription was carried out in a final volume of 25 μl using 2 μg of total RNA and 2.5 units of Superscript II reverse transcriptase (Invitrogen) in the buffer supplied by the manufacturer, in the presence of 10 pmol of oligo(dT)15 and 10 units of RNasin (Promega). PCR was carried out using 2 μl of the RT reaction in the presence of 2 mM dNTPs, 10 pmol of primers, and 2.5 μl of DNA polymerase (Expand high fidelity PCR System; Roche Applied Science). The PCR primers used to verify the alternative splicing of NMHC II-C in human skeletal muscle were 5′-ATGCTGACAGGTGCGAGGAC-3′ (forward) and 5′-ATGAAATT-GCCGAATCGGCGAGG-3′ (reverse).

RNA blot analysis—Multiple Tissue Northern blots and Multiple Tissue Northern blots were used (Molecular Tech, Palo Alto, CA), containing poly(A)- RNA samples from different human and mouse tissues. A PCR product derived principally from the inserted region were as follows: forward primer, 5′-GCCCATGTGG-1979 in mouse); mouse C-terminal sequence, APGQEPEAPPPATPQ-19; see Fig. 1); mouse C-terminal sequence, APGQEPEAPPATPQ (amino acids 1986–2000); and human C-terminal sequence, RGVR-1979 in human). The last of these antibodies appeared to cross-react with a non-muscle filamentous protein in cultured peripheral neurons when used for immunofluorescence studies.7 The mouse sequence antibodies were raised in rabbits by Robert Wysolmerski (St. Louis University School of Medicine, St. Louis, MO) and affinity-purified by us. Antibodies to NMHC II-A and II-B have been described previously (7, 10, 13).

Immunoblot analysis—Tissue extracts from mouse organs were prepared using a buffer of 80 mM MOPS (pH 7.4), 60 mM KCl, 10 mM MgCl2, 5 mM ATP, 4 mM EDTA, 1 mM diethiothreitol, 1% Nonidet P-40, and protease inhibitors (chymostatin, 1-chloro-3-tosylamido-7-aminoc-2-heptanone, t-leucine, and pepstatin A), at 4 °C. The lysates were sedimented at 10,000 × g for 10 min, and the supernatant was used. In samples subjected to immunoprecipitation, the nonmuscle myosins were first purified by actin selection (see below). For immunoprecipitation, a 1:20 (v/v) ratio of affinity-purified antibody to the actin-selected sample was used as described previously (9). Control and relevant immunoprecipitates were fractionated by SDS-PAGE on 6% Tris-glycine gels or 8-16% gradient Tris-glycine gels (Invitrogen), transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA), and subjected to immunodetection using the antibodies described above at a concentration of 0.025 μg/ml for NMHC II-A, 0.045 μg/ml for II-B, and 0.13 μg/ml for II-C. Antibodies to nonmuscle actin (Sigma) and glyceraldehyde-3-phosphate dehydrogenase (Biosdesign, Kennebunk, ME; Ref. 18) were used to assure equal loading of samples. For immunodetection of baculovirus-expressed, mouse FLAG-tagged, heavy meromyosin (HMM) II-C and HMM II-C, both monoclonal anti-FLAG antibody (Sigma) and the polyclonal antibody raised to a sequence near the N terminus of the mouse NMHC II-C were used (see above). Peroxidase-conjugated goat anti-rabbit or anti-mouse IgGs were used as secondary antibodies. The proteins were visualized with the SuperSignal system (Pierce). The protein concentrations were determined using a Bio-Rad protein assay kit.

Immunofluorescence microscopy—Distribution of NMHC II isoforms in developing mouse embryos was visualized by immunofluorescence microscopy. The embryos were fixed in phosphate buffered saline and then directly immersed in 4% paraformaldehyde overnight at room temperature. For better fixation, the brain and abdominal cavity were partially exposed. The fixed embryos were embedded in paraffin and sectioned at a thickness of 5 μm. For antibody staining, the samples were blocked with phosphate-buffered saline containing 0.1% bovine serum albumin and 5% normal goat serum for 1 h at room temperature, incubated with polyclonal antibodies against NMHC II-A (0.25 μg/ml), II-B (0.29 μg/ml), and II-C (1.3 μg/ml) overnight at 4 °C, followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch) for 1 h at room temperature. After washing, the coverslips were mounted using a Prolong antifade kit (Molecular Probes). The images were collected using a Leica SP confocal microscope (Leica).

Baculovirus expression of FLAG-tagged HMM II-C and HMM II-C—Plasmid DNA was used to obtain baculovirus recombinants using a FLAG-tagged oligonucleotide primer and cloned into baculovi- rus transfer vector pVL1392 (BD Pharmingen, San Diego, CA). To generate a clone of the 8-amino acid inserted isoform, we used a PCR-derived clone of nucleotides 1–1227 in the TOPO- TA vector (Invitrogen) and introduced the 24-nucleotide cassette insert into it by mutagenesis using site-directed mutagenesis, with the insert (AATGTG) and the 3′ portion of the HMM II-C was then introduced into pVL1392 and the virus was co-transfected along with a virus containing both light chains

2 P. Bridgman, personal communication.
in Sf9 cells (26). Infected cells were harvested by sedimentation after 72 h of growth, and the pellet was washed twice with phosphate-buffered saline, quick-frozen in liquid nitrogen, and stored at -80°C.

Extraction and purification of HMM was as previously described (27) with the exception that 0.5M NaCl was used in place of 0.2M NaCl. The material eluted from the FLAG column was concentrated using a Sepharose Q column or used directly following elution from the FLAG column.

**Actin Selection of Tissue Extracts and of Baculovirus-expressed Mouse Heavy Meromyosin II-C**

The baculovirus-expressed HMMs were dialyzed overnight in a buffer of 0.5M NaCl, 10 mM MOPS, pH 7.2, 0.1 mM EGTA, 3 mM NaN₃, and 1 mM dithiothreitol (buffer A). F-actin (3 mM), stabilized with 5 mM phalloidin, was added to the expressed HMM, or to lung extracts prepared as outlined above, and the solution was sedimented for 15 min at 470,000 × g to pellet the actomyosin complex. The resulting pellet was solubilized in buffer A containing 5 mM ATP, 5 mM MgCl₂, and 2 mM phalloidin and resedimented to separate the released HMM from the polymerized actin (26).

**ATPase Assay**

The actin-activated MgATPase activity was measured at 37°C using the NADH-coupled assay in a Beckman DU640 spectrophotometer in a buffer containing 2 mM MgCl₂, 0.1 mM EGTA, 1 mM ATP, 0.2 mM CaCl₂, 1 mM calmodulin, and 10 ng/ml myosin light chain kinase as previously described (28).

**In Vitro Motility Assay**

The in vitro motility of the HMM fragments was carried out at 30°C as previously described (29, 30). The conditions were 80 mM KCl, 5 mM MgCl₂, 1 mM ATP, 0.1 mM EGTA, 50 mM dithiothreitol, 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase, and 2 mg/ml catalase. The samples were phosphorylated on the slide as previously described (29).
Identification and Sequencing of Mouse NMHC II-C

Based on the analysis of genomic and cDNA sequence data in the different gene banks, we predicted the amino acid sequences of the human and mouse NMHC II-C in two alternatively spliced forms. We cloned and verified the sequence of the mouse NMHC II-C using RT-PCR and mRNA from mouse lung and colon. Each PCR segment was generated and sequenced at least three times to verify the accuracy of the sequence. The resulting sequence of the cDNA encoding mouse NMHC II-C is presented in Fig. 1 (GenBank™ accession number AY205605, and AY363100 for the alternatively spliced isoform). The protein sequence derived from this cDNA contains the characteristic domains of a myosin II heavy chain (underlined in Fig. 1 and see legend in Ref. 31 to Fig. 1). The cDNA encodes a gene product of 2000 amino acids with the inserted exon and 1992 without it.

Comparisons of the Sequence of NMHC II-C to That of Other Myosin IIs—Comparative analyses of both the phylogenetic tree of the motor domain of NMHC IIs (Fig. 2 and Refs. 24 and 32), as well as the entire heavy chain sequence (Table 1), suggests that NMHC II-C constitutes a distinct branch of the nonmuscle/smooth muscle myosin heavy chain II family. The identity and similarity levels among NMHC II-A (MYH9), NMHC II-B (MYH10), and smooth muscle myosin heavy chain (MYH11) are higher than between any one of them and NMHC II-C. On the other hand, NMHC II-C is more related to this group of myosin heavy chains than to any other myosin heavy chain subfamily (Fig. 2). Furthermore, the percentage of amino acid identity of each myosin isoform between human and...
mouse ranges between 93.3% and 98.7%, which is considerably higher than the percentage of amino acid identity between the different isoforms within the same species.

**NMHC II-C mRNA Tissue Distribution**—RNA blot analysis of human tissue poly(A⁺) RNA with either a probe from the 3′-untranslated region of human NMHC II-C (Fig. 3) or from the 5′ portion of the cDNA (data not shown) showed the presence of a single band of ~7 kb, with highest expression in skeletal muscle. The gene is also expressed in brain, heart, colon, kidney, liver, small intestine, and lung. No detectable message is found in thymus, spleen, placenta, and lymphocytes (Fig. 3). This result is in agreement with the analysis of the tissue distribution of the human mRNA signal using the relative frequency of specific sequences in 3′-directed cDNA libraries (BodyMap website: bodymap.ims.u-tokyo.ac.jp), which also showed that NMHC II-C is most abundant in skeletal muscle (score 4.0) and is absent in thymus, spleen, placenta, and lymphocytes.

Hybridization of a probe from the 3′-untranslated region of human NMHC II-C to a human multiple tissue array blot generally agreed with the results in the RNA blot (Fig. 3) and revealed additional differences between the distribution of NMHC II-C and other nonmuscle NMHC mRNAs (Table II). The table shows that NMHC II-C mRNA is highly expressed in the corpus callosum, which is particularly enriched for glial cells. This finding is confirmed by the BodyMap tissue distribution website (bodymap.ims.u-tokyo.ac.jp), which also shows high levels of this message (GS14638) in the corpus callosum (score = 4.1, which is comparable with a score of 4.0 in skeletal muscle; Fig. 3). Table II also shows that expression of NMHC II-C is low in organs composed mainly of smooth muscle, such as the aorta, uterus, and urinary bladder. NMHCs II-A and II-B are relatively abundant in these tissues (Table II and Ref. 7), whereas the mRNA encoding NMHC II-C is barely detectable. Finally, Table II shows that, in general, human fetal tissue contains little or no NMHC II-C mRNA compared with adult tissues (compare the values for fetal tissue in the last

| Central nervous system | Muscular organs | Other organs | Fetal organs |
|------------------------|----------------|-------------|-------------|
| Whole brain            | Heart: left ventricle | Lung | Fetal lung |
| II-A: 22               | II-A: 85        | II-A: 193  | II-A: 14   |
| II-B: 254              | II-B: 68        | II-B: 129  | II-B: 180  |
| II-C: 82               | II-C: 168       | II-C: 142  | II-C: 2    |
| Frontal lobe           | Heart: left atrium | Liver | Fetal liver |
| II-A: 15               | II-A: 67        | II-A: 68   | II-A: 70   |
| II-B: 207              | II-B: 105       | II-B: 42   | II-B: 92   |
| II-C: 50               | II-C: 86        | II-C: 193  | II-C: 0    |
| Pons                   | Aorta           | Spleen     | Fetal spleen |
| II-A: 11               | II-A: 156       | II-A: 107  | II-A: 240  |
| II-B: 162              | II-B: 504       | II-B: 12   | II-B: 0    |
| II-C: 254              | II-C: 6         | II-C: 0    | II-C: 0    |
| Cerebellum             | Skeletal muscle | Thymus     | Fetal Thymus |
| II-A: 19               | II-A: 29        | II-A: 192  | II-A: 193  |
| II-B: 74               | II-B: 4         | II-B: 0    | II-B: 0    |
| II-C: 71               | II-C: 362       | II-C: 11   | II-C: 0    |
| Corpus callosum        | Urinary bladder | Colon (transverse) | Fetal brain |
| II-A: 26               | II-A: 268       | II-A: 450  | II-A: 14   |
| II-B: 33               | II-B: 320       | II-B: 57   | II-B: 180  |
| II-C: 362              | II-C: 388       | II-C: 9    | II-C: 2    |
| Thalamus               | Uterus          | Kidney     | Fetal Heart |
| II-A: 13               | II-A: 282       | II-A: 106  | II-A: 109  |
| II-B: 123              | II-B: 174       | II-B: 107  | II-B: 30   |
| II-C: 81               | II-C: 4         | II-C: 117  | II-C: 4    |

**Fig. 4. Ratio of inserted to noninserted mRNAs in mouse tissues.** RT-PCR products are shown for the indicated tissues following ethidium bromide staining and gel electrophoresis. The inserted isoform migrates more slowly than the noninserted one. The bp sizes of the markers are given on the right, and the sizes of the products are on the left.

**Fig. 5. Immunoblot analysis following immunoprecipitation of NMHC II-C from an actin-selected myosin preparation from lung.** The starting material was sedimented from a lung extract using F-actin and released into the supernatant with MgATP. All three myosins are present in the actin-selected material (lanes 1, 4, and 7). Only NMHC II-C is precipitated by the antibody to II-C (compare lane 9 with lanes 3 and 6). The antibody used for immunoprecipitation was raised to the C-terminal mouse myosin II-C sequence. The other antibodies for immunoblot analysis (II-A and II-B) are described previously (7). Antibodies used for immunoblotting are indicated below the panels. IP, immunoprecipitate.

**Table II**

| Levels of NMHC II-A, II-B, and II-C mRNA in various human organs, using the multiple tissue array (Clontech) |
|---------------------------------------------------|-------------------------------------------------|----------------|----------------|
| The data are adjusted to the signals generated by a ubiquitin probe and normalized so that the value of 100 defines the median ratio of the signals of 12 different tissues of each NMHC (brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, placenta, uterus, lung, and peripheral blood lymphocytes). |-------------------------------------------------|----------------|----------------|

The table shows that NMHC II-C mRNA is highly expressed in the corpus callosum, which is particularly enriched for glial cells. This finding is confirmed by the BodyMap tissue distribution website (bodymap.ims.u-tokyo.ac.jp), which also shows high levels of this message (GS14638) in the corpus callosum (score = 4.1, which is comparable with a score of 4.0 in skeletal muscle; Fig. 3). Table II also shows that expression of NMHC II-C is low in organs composed mainly of smooth muscle, such as the aorta, uterus, and urinary bladder. NMHCs II-A and II-B are relatively abundant in these tissues (Table II and Ref. 7), whereas the mRNA encoding NMHC II-C is barely detectable. Finally, Table II shows that, in general, human fetal tissue contains little or no NMHC II-C mRNA compared with adult tissues (compare the values for fetal tissue in the last

...
Myosin II-C, a New Nonmuscle Myosin

2805

Procedures

Fig. 7 shows the distribution of the inserted and noninserted mRNA in a variety of mouse tissues. The figure shows that although the inserted mRNA is the predominant one in adult mouse liver, kidney, and testis, brain and lung contain approximately equal amounts of both mRNAs, and skeletal muscle and heart contain very low amounts of the inserted message. Spleen is a negative control, because NMHC II-C is not expressed in this organ. Comparison of the inserted amino acid sequence between mice and humans showed a difference in one amino acid (ASVSTMSY for mice and ASVSTVSY for humans).

NMHC II-C Protein Is Detected by Specific Antibodies—The selectivity of the antibody raised to the mouse C-terminal sequence is demonstrated in Fig. 5. This figure shows an immunoblot of a crude myosin fraction containing all three nonmuscle myosin II isoforms prepared by actin selection from mouse lungs (lanes 1, 4, and 7; see “Experimental Procedures” and Ref. 26). Only NMHC II-C is immunoprecipitated using the antibody raised to the II-C peptide as shown by immunoblotting the immunoprecipitate with antibodies to II-A, II-B, and II-C (Fig. 5, lanes 3, 6, and 9). The absence of NMHC II-A and II-B from the immunoprecipitate also shows that NMHC II-C is a homodimer with respect to the myosin heavy chains rather than a heterodimer of II-C with either II-A or II-B.

Fig. 6 is an immunoblot comparing the protein expression of NMHC II-A, II-B, and II-C. Note that two separate blots are included for each panel: one utilizing 10 tissue samples and one with three. Equal amounts of protein, as determined by Coomassie Blue staining and detection of nonmuscle actin (left panels) and glyceraldehyde-3-phosphate dehydrogenase by antibodies (right panels), were used to assure equal protein loading (data not shown). The blots show that NMHC II-C differs from II-A in being low or nondetectable in seminal vesicles, liver, bladder, and spleen. The presence of NMHC II-C in stomach and colon and its absence in bladder distinguishes its expression from II-A in being low or nondetectable in seminal vesicles, liver, bladder, and spleen. The selective expression of NMHC II-C in colon and stomach in contrast to II-A and its presence in colon and stomach in contrast to II-B. MW indicates molecular mass markers.

Differences in the Localization of the Three NMHCs—Immunofluorescence microscopy was used to examine the distribution of NMHC II-C, II-B, and II-A in mouse embryos at E11.5 and E16.5. Fig. 7 (A, E, and I) shows the widespread distribution of all three isoforms in the mouse embryo. (The apparent concentration of II-A and II-C in the liver at E11.5 most likely is due to autofluorescence secondary to the presence of blood cells.) Panels B, F, and J of Fig. 7 are enlargements of the mouse brains from the previous sections as indicated. Note the enhanced staining for II-C in the developing pituitary at E11.5 (Fig. 7B), for II-B at the pial and ventricular surfaces of the brain (Fig. 7F), and for II-A in the vasculature (Fig. 7J).

Panels C, G, and K of Fig. 7 show differences in the localization of the isoforms in the inner ear. Fig. 7C shows intense staining for II-C in the developing sensory area of the cochlea, whereas II-B is more widely expressed in the epithelial cells and the surrounding mesenchymal cells (Fig. 7G). Again the staining for II-A is most intense in the vessels, although clearly the isoform is also present in both the epithelial and mesenchymal cells (Fig. 7K). Panels D, H, and L of Fig. 7 show that II-C staining is most intense in the apical area of the epithelial cells in the E16.5 mouse intestine (Fig. 7D), and, although staining for II-A is also relatively intense in these cells, it is not localized to the apical part of the cell, as is II-C (7L). Fig. 7H shows that II-B stains most intensely in the serosal cells surrounding the intestine but not in the epithelial cells.

Expression of Recombinant FLAG-tagged HMM II-C and HMM II-C1—We used the Baculovirus expression system to generate both alternatively spliced forms of FLAG-tagged heavy meromyosin II-C along with the 20- and 17-kDa myosin light chains. Both HMM II-C and the 8-amino acid inserted isoform HMM II-C1 bound to skeletal muscle actin and detached from it in the presence of MgATP (shown for HMM II-C in Fig. 8A, lane 4). The figure shows a Coomassie Blue-stained gel depicting purified HMM II-C (Fig. 8A, lane 4) and purified HMM II-C1 (Fig. 8B, lanes 3–8). In this case, the HMM II-C1 was used for characterization studies directly after the FLAG column, although in some cases, it was subjected to actin selection prior to characterization. The finding that both HMM II-C and II-C1 were capable of binding to actin in the absence of MgATP and were released in the presence of MgATP demonstrates that both isoforms share a basic biological property of myosins. However, despite repeated efforts, we were only able to obtain sufficient quantities of the inserted isoform, HMM II-C1, for characterization of the actin-activated MgATPase
activity and in vitro motility assay. These values are shown in Table III and are compared with those obtained previously for NMHC II-B and II-A HMM. The table shows that HMM II-C has a $V_{\text{max}}$ between that of II-B and II-A and translocates actin filaments at approximately the same rate as HMM II-B, considerably more slowly than HMM II-A.

**DISCUSSION**

In this study, we present evidence for a previously unrecognized myosin II that is expressed at the protein level in both muscle and nonmuscle tissues. We refer to the new myosin heavy chain as NMHC II-C. We present here the nucleotide and derived amino acid sequence of two alternatively spliced forms of NMHC II-C, which contains all the characteristic domains of conventional myosin heavy chains. Using specific antibodies, we provide evidence that this sequence is expressed as a protein.

Recently, Leal et al. (25) have studied the region of the human chromosome 19q13.3 and have reported the genomic structure and mRNA distribution of NMHC II-C, as evidenced...
by an RNA blot. Their computer data analysis overlooked the alternative spliced variant NMHC II-C1 described above but suggested the existence of shorter alternative spliced forms of this myosin lacking major components of the motor domain. In our analysis, we found similar data but could not confirm it by cloning. Our RNA distribution analysis suggests that NMHC II-C is more widely distributed than the presentation by Leal et al. (25).

Fig. 3 provides a comparison of the results of two different approaches to determine the level of expression of NMHC II-C mRNA: the classical approach of an RNA blot and the emerging approach of estimating the level of certain mRNAs according to the number of specific gene tags in a 3'-directed cDNA library. Information about gene expression patterns using the latter approach is available from different websites for many genes, including ones that are not yet identified and characterized. In both approaches, we found that NMHC II-C is abundantly expressed in human skeletal muscle and absent in spleen, thymus, placenta, and lymphocytes. With the exception of the expression level in the lung, the ranking of the expression levels in both approaches is similar, although the ratios among them are different. Given the progress in cDNA data accumulation, the information about gene expression is becoming more accurate, and we show that one can gain valuable information about patterns of gene expression from these data bases.

We further examined the patterns of gene expression of NMHCs in human tissues at the RNA level using multiple tissue arrays and developed a simple approach to compare patterns of expression of the different NMHCs (Table II). Because probes of different genes vary in the efficiency of hybridization, the absolute levels of mRNAs cannot be compared. However, the tissue distribution of mRNA levels around the mean or median can serve as a tool for comparison among different genes in a quantitative manner. Of note is that the data shown in Table II for NMHC II-A and II-B are in agreement with that previously reported based on standard techniques (3–7).

The tissue distribution of NMHC II-C is qualitatively different from other myosin II heavy chains in several respects and suggests that it has a different role from other cytoskeletal myosins. First, NMHC II-C is more abundant in human adult tissues than in human fetal tissues. Unlike NMHC II-A and II-B, it is not expressed in mouse embryonic stem cells. Furthermore, Buxton et al. (33) found that the differentiation stimulus of histone hyperacetylation induces the expression of NMHC II-C, which further supports the idea that myosin II-C is more abundant in differentiated tissues. In addition, NMHC II-C is abundant in specific human brain areas (corpus callosum and pons; Table II) that are rich in glial cells and fiber tracts compared with neuronal cell bodies. On the other hand, the levels of NMHC II-C are very low in organs with abundant smooth muscle, such as uterus, urinary bladder, and aorta, distinguishing it from other nonmuscle myosin IIs.

Nonmuscle myosin II-C behaves differently from most housekeeping genes including NMHC II-A and II-B as shown by its low expression in early development and the fact that, in some instances, it is an inducible protein (33). Other myosins that are known to be induced are cardiac myosins. Two cardiac myosin heavy chains have been described in the heart: α- and β-myosin heavy chains. Their composition is affected by various physiological stimuli, such as pressure overload and endocrine, paracrine, and autocrine stimuli (34–36). Significant alterations in cardiac function are associated with isoform shifts, evidenced by changes in the maximum unloaded shortening velocity and ATP consumption by the contractile machinery (34). This is consistent with the different mechanical and enzymatic properties of the two isoforms (1, 37, 38).

The kinetic mechanisms for NMHC II-A and NMHC II-B are significantly different and suggest that these two myosins probably do not have redundant functions (28, 39, 40). NMHC II-A behaves as a normal but slow conventional myosin with a low duty cycle, meaning that it spends most of its kinetic cycle detached from actin (39). In contrast, NMHC II-B has a duty cycle intermediate between that of conventional and processive unconventional myosins such as myosin V (28, 40). This behavior suggests that NMHC II-B may be better adapted for maintaining tension in a static manner. The kinetic mechanism for NMHC II-C remains to be examined. As expected, both the in vitro motility and the actin-activated MgATPase activity of HMM II-C1 were dependent on phosphorylation of the 20-kDa myosin light chain.

Surprisingly, there was a big difference in the yield of the baculovirus expression of the two alternatively spliced forms of HMM II-C. Although we produced the inserted form by splicing in the insert to a clone of the noninserted form and confirming the sequence afterward, the longer form consistently yielded significantly more protein product, which enabled a more thorough biochemical characterization. The effect of small changes of nucleotide sequence on expression level has been observed before (29).

Unlike cardiac myosins, which form both homodimers (known as V1 and V3) and heterodimers (known as V2) (34), nonmuscle myosins form only homodimers (11). This is exemplified for NMHC II-C since, following immunoprecipitation of NMHC II-C, no NMHC II-A or II-B was found in the precipitate (Fig. 5).

The term “nonmuscle myosin” was generally used to distinguish between the ubiquitous forms of myosin II and the muscle-specific isoforms. However, this term is misleading because these myosins are not only expressed in all types of nonmuscle tissue but also have a significant role in the development and function of muscle tissues such as the heart and smooth muscles (13, 17, 41, 42). Fallon and Nachmias (43) described this inadequacy in myosin nomenclature as early as 1980 and suggested the term “cytoplasmic myosin.” However, this term would preclude the possibility that some of these myosins could also localize to the nucleus and does not differentiate these myosins from sarcomeric myosins. Because these myosins are likely to be involved in determining cell shape and polarity and interact with cytoskeletal actin, we suggest the use of the term cytoskeletal myosin II. Still, no name, at present, is without particular note at E11.5 is the prominence of NMHC II-C in mouse

Table III: Summary of MgATPase and in vitro motility assays

| Reaction        | Vmax       | Kmax       | IVM         |
|-----------------|------------|------------|-------------|
| MgATPase        | s⁻¹        | μM         | μM/s        |
| II-C            | 0.55 ± 0.18| 16.5 ± 7.2 | 0.05 ± 0.011 (−T) |
| II-B (26)       | 0.28 ± 0.08| 12.7 ± 4.1 | 0.077 ± 0.013 (+T) |
| II-B1 (26)      | 0.37 ± 0.09| 15.1 ± 4.5 | 0.092 ± 0.013 (−T) |
| II-A (46)       | 1.2 ± 0.3  | 7.4 ± 1.3  | 0.29 ± 0.03 (−T) |

* C1 and B1 = inserted isoform.

3 M. A. Conti, unpublished observation.
pituitary, the enrichment of NMHC II-B in the brain, particularly at the ventricular and pial cortical areas and the prominence of NMHC II-A in the vasculature of the brain (Fig. 7, B, F, and J). Analysis of the inner ear and epithelial cells of the intestines also showed major differences in the distribution of the three isoforms but clearly indicate cells where the distribution also overlaps (Fig. 7, C, G, and K; D, H, and L). Indeed, very few cells are actually devoid of any one isoform. For example, neuronal cells do contain some NMHC II-A (Fig. 7J and Ref. 44). Importantly, however, the presence of both NMHC II-A and II-C in neuronal cells does not compensate for the loss of II-B following abortion of this isoform (17, 19) or lowering NMHC II-B to less than 20% of the normal amount (18). Moreover, mutation of single amino acids in NMHC II-A and II-C in neuronal cells does not compensate for the consequences of mutation in NMHC II-C in both humans and mice.

Acknowledgment—We thank Robert Wysolmerski for help with raising antibodies, Fei Wang and Estelles Harvey for help in the baculovirus expression, and Antoine Smith, Yael Bromberg, and Joshua Zuckerman for technical assistance. We also thank Catherine Magruder for editorial assistance.

REFERENCES

1. Sellers, J. R. (1999) Myosins, Oxford University Press, Oxford, UK
2. Bresnick, A. R. (1999) Curr. Opin. Cell Biol. 11, 26–33
3. Shohat, R. V., Conti, M. A., Kawamoto, S., Preston, Y. A., Brill, D. A., and Adelstein, R. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7726–7730
4. Katsumura, Y., Yanagisawa, M., Inoue, A., and Masaki, T. (1989) Eur. J. Biochem. 184, 611–616
5. Simons, M., Wang, M., McBride, O. W., Kawamoto, S., Yamakawa, K., Gudla, D., Adelstein, R. S., and Weir, L. (1991) Circ. Res. 69, 530–539
6. Kawamoto, S., and Adelstein, R. S. (1991) J. Cell Biol. 112, 915–924
7. Phillips, C. L., Yamakawa, K., and Adelstein, R. S. (1995) J. Muscle Res. Cell Motil. 16, 379–389
8. Takahashi, M., Kawamoto, S., and Adelstein, R. S. (1992) J. Biol. Chem. 267, 17864–17871
9. Itoh, K., and Adelstein, R. S. (1995) J. Biol. Chem. 270, 14533–14540
10. Maupin, P., Phillips, C. L., Adelstein, R. S., and Pollard, T. D. (1994) J. Cell Biol. 127, 3077–3090
11. Kelley, C. A., Sellers, J. R., Gard, D. L., Bui, D., Adelstein, R. S., and Baines, I. C. (1996) J. Cell Biol. 134, 675–687
12. Kolesa, J. (1998) J. Cell Sci. 111, 2085–2095
13. Takeda, K., Yu, Z.-X., Quan, S., Chinn, T. K., Adelstein, R. S., and Ferrans, V. J. (2000) Cell Motil. Cytoskeleton 46, 59–68
14. Murakami, N., Trenkner, E., and Elzinga, M. (1993) Dev. Biol. 157, 19–27
15. Wylie, S. R., Wu, P. J., Patel, H., and Chantler, P. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12967–12972
16. Wylie, S. R., and Chantler, P. D. (2001) Nat. Cell Biol. 3, 88–92
17. Tullis, A. N., Arcilla, D., Ferrans, V. J., Yu, Z.-X., Takeda, K., Grinberg, A., Westphal, H., Preston, Y. A., and Adelstein, R. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12407–12412
18. Uren, D., Hwang, H.-K., Hara, Y., Takeda, K., Kawamoto, S., Tullis, A. N., Yu, Z.-X., Ferrans, V. J., Tresser, N., Grinberg, A., Preston, Y. A., and Adelstein, R. S. (2000) J. Clin. Invest. 105, 663–671
19. Tullis, A. N., Bridge, P. C., Tresser, N. J., Chan, C.-C., Conti, M. A., Adelstein, R. S., and Hara, Y. (2001) J. Comp. Neurol. 433, 62–74
20. Huang, H., Palouzas, M., Rambaldi, I., Lasko, P., and Featherstone, M. (2003) Mol. Cell Biol. 23, 3636–3645
21. Obinou, V. H., Burgo, A. L., Agarwal, S. K., Chandrasekharapla, S. C., Adelstein, R. S., and Marx, S. J. (2003) Oncogene 22, 6347–6356
22. Roy, M., Vicente-Manzanares, M., Viedma, F., Yanes-Mo, M., Urrainzai, A., Barreiro, O., Vazquez, J., and Sanchez-Madrid, F. (2002) J. Immunol. 169, 5410–5414
23. Ford, H. L., Silver, D. L., Kachar, B., Sellers, J. R., and Zain, S. B. (1997) Cytobiology 36, 16321–16327
24. Berg, J. S., Powell, B. C., and Cheney, R. E. (2001) Mol. Biol. Cell 12, 780–794
25. Leal, A., Endele, S., Stengel, C., Huchne, K., Loetterle, J., Barrantes, R., Winterpach, A., and Rausenstraubs B. (2003) Gene (Amst.) 312, 165–171
26. Pato, M. D., Sellers, J. R., Preston, Y. A., Harvey, E. V., and Adelstein, R. S. (1996) J. Biol. Chem. 271, 2689–2695
27. Wang, F., Chen, L., Arcuolo, O., Harvey, E. V., Bowers, B., Xu, Y., Hammer, J. A., and Sellers, J. R. (2000) J. Biol. Chem. 275, 4329–4335
28. Wang, F., Kovacs, M., Hu, A., Limouze, J., Harvey, E. V., and Sellers, J. R. (2003) J. Biol. Chem. 278, 27439–27448
29. Hu, A., Wang, F., and Sellers, J. R. (2002) J. Biol. Chem. 277, 46512–46517
30. Sellers, J. R., Cuda, G., Wang, F., and Homsher, E. (1993) Methods Cell Biol. 39, 25–49
31. Reeves, M. A., and Holmes, K. C. (1999) Annu. Rev. Biochem. 68, 687–697
32. Cheney, R. E., Riley, M. A., and Mooseker, M. S. (1993) Cell Motil. Cytoskeleton 24, 215–223
33. Buxton, D. B., Golomb, E., and Adelstein, R. S. (2003) J. Biol. Chem. 278, 15449–15455
34. Litten, R. Z., Martin, B. J., Lov, R. B., and Alpert, N. R. (1982) Circ. Res. 50, 856–864
35. van Buren, P., Harris, D. E., Alpert, N. R., and Warshaw, D. M. (1995) Circ. Res. 77, 439–444
36. Ng, W. A., Grupp, I. L., Subramaniam, A., and Robbins, J. (1991) Circ. Res. 68, 1742–1750
37. Sata, M., Sugiyama, S., Yamashita, H., Momomura, S., and Serizawa, T. (1993) Circ. Res. 73, 696–704
38. Cuda, G., Fute, E., Cooke, R., and Sellers, J. R. (1997) Biophysical J. 72, 1767–1779
39. Kovacs, M., Wang, F., Hu, A., Zhang, Y., and Sellers, J. R. (2003) J. Biol. Chem. 278, 38132–38140
40. Rosenfeld, S. S., Xing, J., Chen, L. Q., and Sweeney, H. L. (2003) J. Biol. Chem. 278, 27449–27455
41. Morano, I., Chai, G. C., Baltas, L. G., Lamouneur-Zepter, V., Lutsch, G., Kott, M., Haase, H., and Bader, M. (2000) Nat. Cell Biol. 2, 371–375
42. Du, A., Sanger, J. M., Linask, K. K., and Sanger, J. W. (2003) Dev. Biol. 257, 382–394
43. Fallon, J. R., and Nachmias, V. T. (1980) J. Cell Biol. 86, 2085–2103
44. Schmehl, A. M., Trenkner, E., Elzinga, M., and Wysolmerski, R. (1989) J. Cell Sci. 94, 2095–2105
45. Wang, F., Harvey, E. Y., Conti, M. A., Wei, D., and Sellers, J. R. (2000) Biochemistry 39, 5555–5560

4 X. Ma, unpublished results.