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Genome Structure and Differential Expression of Two Isoforms of a Novel PDZ-Containing Myosin (MysPDZ) (Myo18A)

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We previously cloned a gene for a novel myosin (called MysPDZ) containing a PDZ-domain from bone marrow stromal cells. This new myosin is found in humans and classified as one of the class XVIII myosins (Myo18A). Here, we report the hematopoietic cell-specific splicing isoform (MysPDZβ) in addition to the previously reported isoform (MysPDZα). Combined with mouse genome sequence data, the overall genome structure and generation of the two spliced isoforms are deduced. The MysPDZβ protein lacks a PDZ-domain in the N-terminal region. Studies of the subcellular localization of the two spliced isoforms indicated that MysPDZα containing the PDZ domain co-localizes with the ER-Golgi complex, while MysPDZβ, which lacks the PDZ domain, localizes diffusely in the cytoplasm. These results suggest that the isoforms differ in their subcellular localization and may have different functions in membrane ruffling and membrane traffic pathways. The PDZ-containing spliced isoform (MysPDZα) is not expressed in bone marrow hematopoietic cells, whereas MysPDZβ lacking the PDZ is specifically expressed in most hematopoietic cells. It is noted that neither isoform is expressed in red blood cells. Interestingly, MysPDZα was detected in mature but not in immature macrophages, and its level increased after the induction of differentiation of M1 cells, suggesting a functional role of PDZ-containing myosin in macrophages.

Key words: hematopoietic cells, isoforms, new myosin, PDZ domain.

We earlier cloned a new gene for a novel myosin, called MysPDZ, whose expression levels correlate with the hematopoietic supportive ability of stromal cells (*1*). This novel myosin has an N-terminal PDZ domain, one IQ motif, and a tail of segmented coiled-coil. As an unconventional myosin it is intriguing, because unconventional myosins are involved in many cellular processes: cell growth and development, cell movement, RNA transport, organelle transport, particle movement, endocytosis, and exocytosis (*2–5*). It has also recently been learned that clustering and site-specific localization of membrane molecules are important for cell to cell communication, and these events are regulated by scaffolding proteins such as PDZ containing proteins (*6*). Multiprotein complexes that include PDZ containing proteins are anchored to cytoskeletal filaments such as microtubules (*7*, *8*) and actin filaments (*9*, *10*), and this anchoring is necessary to enlarge the stability and signal transduction of cell to cell contact. Thus MysPDZ may be involved in the maintenance of the stromal cell architecture required for cell to cell contact by hematopoietic cells.

This novel myosin was classified as a class XVIII myosin in recent reviews (*11*, *12*), and is found in vertebrates and *Drosophila* (*12*, *13*) with strong conservation, although it is not found in *C. elegans*, *Arabidopsis thaliana*, *Dictyostelium* or *S. cerevisiae*. Thus, myosins containing an N-terminal PDZ domain constitute a new class of myosins, class XVIII, and are thought to be of phylogenetic functional importance.

This genetic information also suggests the presence of spliced isoforms of MysPDZ lacking the PDZ domain. In addition, several classes of unconventional myosins have alternative splicing isoforms, including mouse myosin V (*14*, *15*), human and mouse myosin VIIA (*16–18*), and myo IXA/myr7 (*19–21*), and these have different distributions and functions. Myosin I, a single-headed member of the myosin superfamily, is known to be widely distributed in metazoan cells. There are at least six different subclasses of myosin I proteins (*11*, *22*). Thus, it is important to find such spliced isoforms and analyze their functions to learn the biological function of this new type myosin. In the present study, we deduced the overall genome structure of MysPDZ (Myo18A) and found a new isoform (MysPDZβ) lacking the PDZ domain expressed specifically in hematopoietic cells, in addition to the previously identified stromal form (MysPDZα). We also demonstrated that both isoforms differ in their subcellular localization and suggest that they may have different functions in membrane ruffling and membrane traffic pathways.

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MATERIALS AND METHODS

Northern Blotting—RNAs extracted from mouse tissues were separated in 1% agarose gels and transferred to nylon membranes. Following prehybridization, the blots were hybridized overnight in hybridization buffer with ^{32}P-labeled probes prepared from a cDNA fragment.

Cloning of cDNA for MysPDZβ Spliced Isoforms—To isolate the MysPDZβ specific 5′-sequence, its cDNA was cloned by PCR amplification using a mouse spleen cDNA

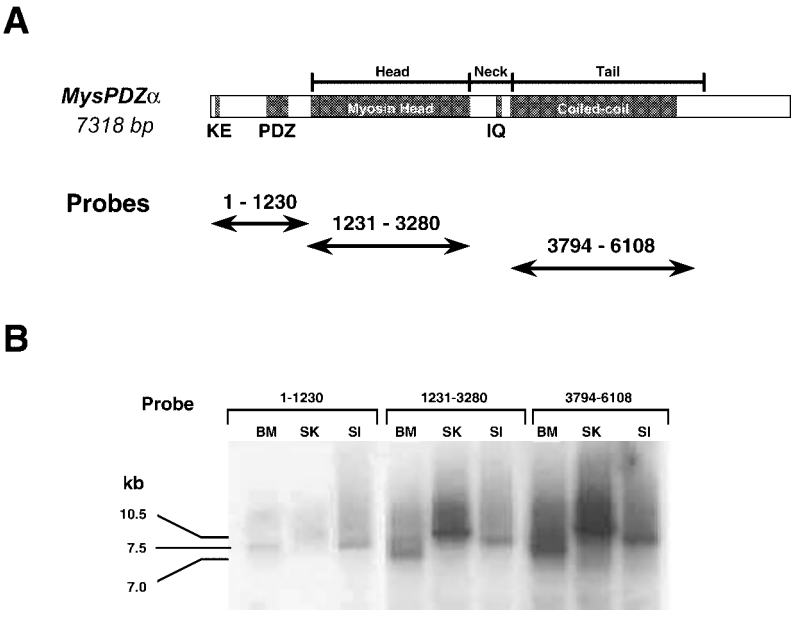
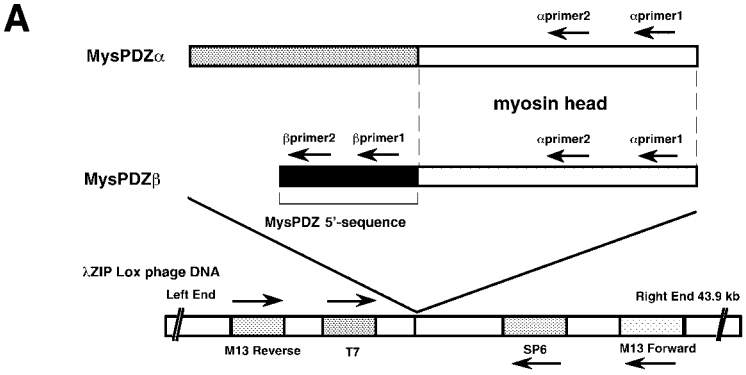


Fig. 1. **Differences between the tissue-specific spliced isoforms of MysPDZ mRNA.** (A) Schematic diagram of MysPDZα mRNA and regions of the ^{32}P-labeled probes [1-1230 nt (ORF containing the KE-rich sequence and PDZ-domain), 1231–3280 nt (ORF containing the Head domain), and 3794–6108 nt (ORF of the Neck and Tail domains)] prepared from a cDNA fragment for Northern blot hybridization. (B) Tissue specific spliced isoforms of MysPDZ. Three different probes hybridize with 7.5 kb, 7.0 kb, and 10.5 kb MysPDZ mRNA, but, in the bone marrow, the nucleotide comprised of 1–1230 does not hybridize with the 7.0 kb splicing isoform of MysPDZ. BM; bone marrow, SK; skeletal muscle, SI; small intestine.



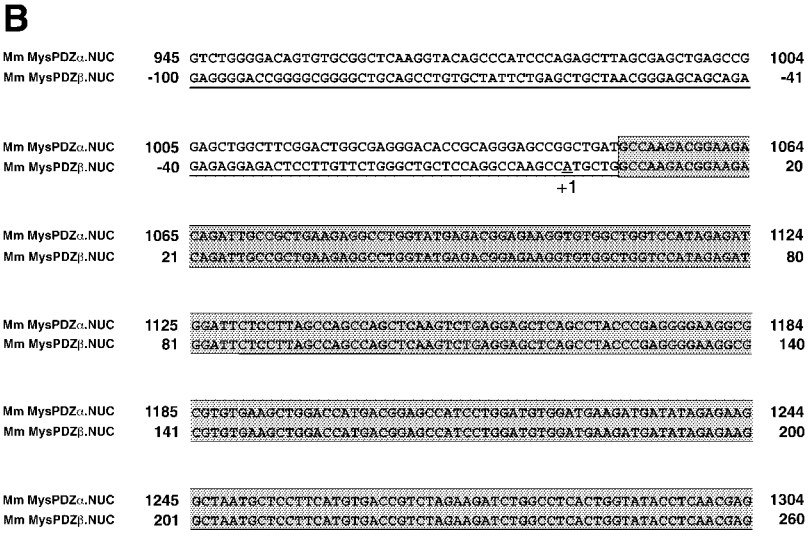


Fig. 2. **Isolation of the MysPDZβ-specific 5′-sequence.** (A) To isolate the MysPDZβ-specific 5′-sequence, its cDNA was cloned by PCR amplification using a mouse spleen cDNA library (λZIP Lox phage DNA). MysPDZβ cDNA was amplified using two primers (αprimer1, αprimer2) as lower primers from the myosin homology region of MysPDZα, and M13 Forward, M13 Reverse, or SP6, T7 primer as upper primers located in the λZIP Lox phage arms. The amplified cDNA was sequenced and other primers (βprimer1, βprimer2) within the newly extended sequences were used to determine of further 5′-sequences. The MysPDZβ-specific 5′-sequence was finally determined by the 5′ RACE method. (B) The cloned MysPDZβ-specific 5′-sequence comprised 106 nucleotides, extending from the terminus of the myosin homologous region. The newly determined sequence lacked the KE-rich sequence and PDZ domain, differing completely from the 5′-terminus of MysPDZα. (C) Schematic diagram of the MysPDZ genomic structure responsible for generating isoforms. The genome structure of mouse (Myo18A) was predicted from genome annotations, to comprise of 40 exons and to generate at least 2 spliced isoforms (mmMysPDZ α and mmMysPDZβ). The genome structure of human MysPDZ (MYO18A) was also predicted from genome annotations. Human MysPDZα (HsMysPDZα) was predicted from the nucleotide sequences of KIAA0216 and D86970 and is equivalent to that of mouse mmMysPDZα. Human MysPDZβ was predicted from the nucleotide sequences of KIAA0216 and is equivalent to that of mmMysPDZβ.

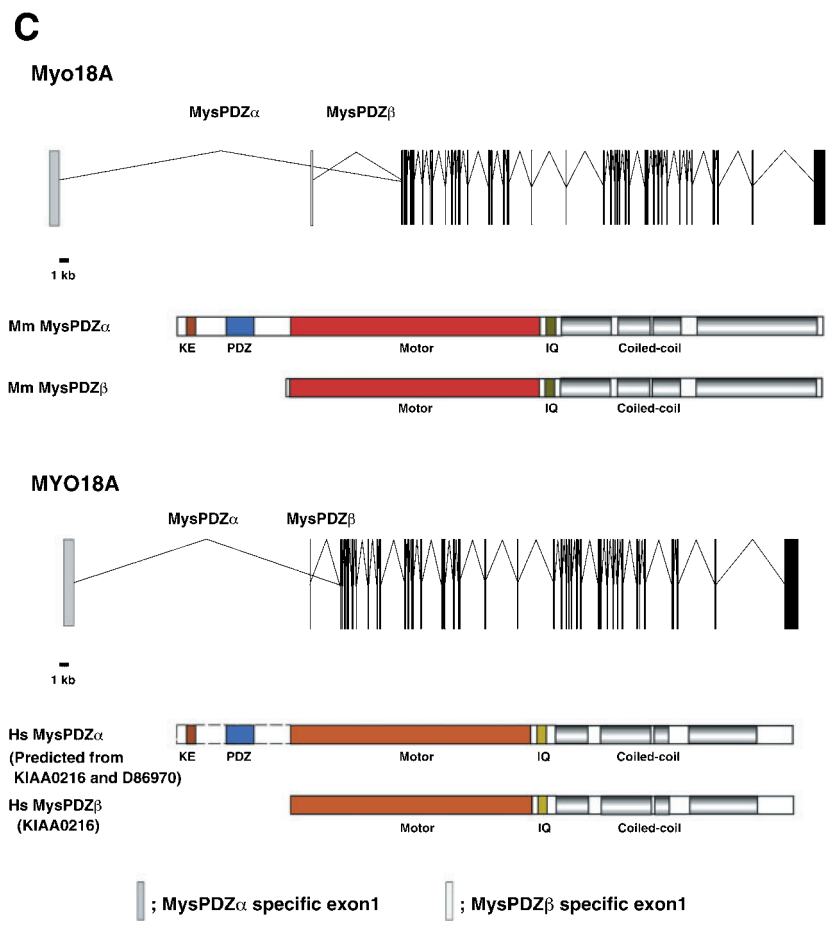


Fig. 2. (C)

library (λZIP Lox phage DNA). The cloning strategy is described in Fig. 2A in detail. To determine the specific 5′-sequence, PCR was applied to a λZIP Lox C57BL/6 mouse spleen cDNA library using primers (αprimer1; 5′-GGACGCTAGACTCGTTGAGG-3′, αprimer2; 5′-AGGCCAGATCTTCTAGACGG-3′) located in the myosin homology region of MysPDZα, and M13reverse (5′-GGAAACAGCTATGACCAT-3′) or M13 forward (5′-GTAAAACGACGCCAG-3′) primers and SP6 (5′-CATACGATTTAGGTGACACTATAG-3′) or T7 (5′-TAATACGACTCACTATAGGG-3′) primers located in the λZIP Lox phage arms. An amplified cDNA fragment was subcloned into pBluescript II KS(+) vector (STRATAGENE, LA Jolla, CA) by the TA-cloning method, and the sequence of the 5′ region was determined by nucleotide sequencing. DNA sequencing was performed with an automatic sequencer (ABI PRISM sequencer 377, ABI). To identify the 5′-terminal coding region, a 5′ RACE procedure was employed using a SMART RACE cDNA Amplification Kit (CLONTECH).

Cell Culture—Macrophage cell line J774.1, and mouse myeloma cell line NS-1 were cultured in RPMI1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS). PreB cell lines 70Z/3 and 18–81, mature B cell line M12, and thymocyte cell line EL-4 were cultured in RPMI1640 (Sigma) supplemented with 10% FBS and 0.1 mM 2-mercaptoethanol. Mouse myeloid leukemia cell line M1 was cultured in DME (Sigma) supplemented with 10% heat-inactivated horse serum.

Detection of MysPDZ mRNA Isoforms by RT-PCR—Total RNA was isolated by an acid phenol procedure using ISOGEN (Wako Pure Chemicals, Tokyo) according to the manufacturer's protocol. Two micrograms of total RNA was reverse-transcribed with SuperScript II RT (GIBCOBRL, Grand Island, NY), and the cDNAs were amplified with 0.05 U/ml ExTaq polymerase (TaKaRa, Tokyo) and primers (MysPDZα primers; 5′-AGATGATCCGGCAGTCTGG-3′ and αprimer2, MysPDZβ primers; 5′-GCTGCAGCCTGTGCTATTC-3′ and αprimer2, myosin head primers; 5′-CTCTTCAGCGGCAATCTGCT-3′ and αprimer2, β-actin primers; 5′-CCTAAGGCCAACCGTGAAAAG-3′ and 5′-TCTTCATGGTGCTAGGAGCCA-3′). Cycling conditions were 30 cycles (for MysPDZα, MysPDZβ, myosin head) or 25 cycles (β-actin) consisting of successive incubations at 94°C (30 s), 60°C for MysPDZα, MysPDZβ, myosin head (30 s) or 55°C for β-actin (30 s), and 72°C (60 s). The amplified PCR products were analyzed by 2% agarose gel electrophoresis.

Preparation of Normal Hematopoietic Cells—Bone marrow cells were flushed from the femurs of mice (C57BL/6) with Dulbecco's phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin and 5 mM EDTA (PBS-BSA-EDTA). The cell suspension was washed and passed through nylon mesh (Falcon 2350, Becton Dickinson, Lincoln Park, NJ) to make a single cell suspension. Red blood cells were lysed with lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA). The hematopoietic cell fractions were incubated with mono-

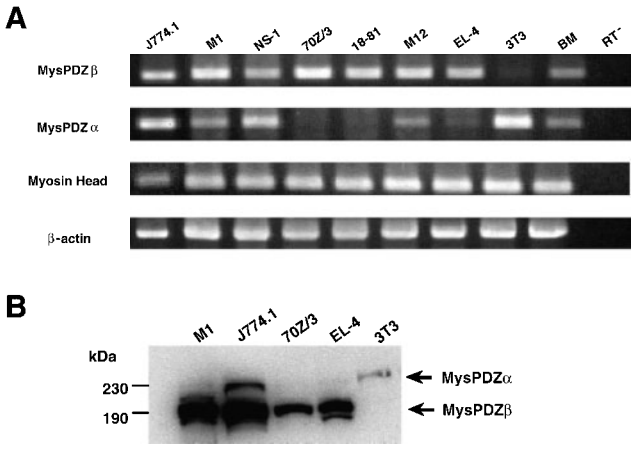


Fig. 3. **Expression of two isoforms, MysPDZα and MysPDZβ, in hematopoietic cells.** (A) RT-PCR analysis of MysPDZ mRNA in hematopoietic cell lines. Expression of two isoforms in hematopoietic cells examined using several murine hematopoietic cell lines (J774.1; macrophage cell line, M1; myeloid leukemia, NS-1; myeloma cell line, 70Z/3 and 18–81; preB cell lines, M12; mature B cell line, EL-4; thymocyte cell line). MysPDZβ mRNA was detected in all hematopoietic cell lines and bone marrow cells (BM). Some hematopoietic cell lines (J774.1, M1, NS-1, M12) showed MysPDZα mRNA expression. (B) Expression of the MysPDZ isoform proteins. The expression of the MysPDZ isoform proteins was confirmed by Western blot analysis using anti-MysPDZ antibody. MysPDZα was detected as a 230 kD protein in fibroblast cell line 3T3. 70Z/3 and EL-4, which expressed only MysPDZβ mRNA, showed most protein bands with a molecular size of 190 kD, whereas J774.1, which expressed the mRNA of both isoforms showed the 230 kD band in addition to the 190 kD band.

clonal antibodies specific to lineage markers (anti-B220: RA3-6B2, PharMingen, San Diego, CA; anti–Mac-1: M1/70, Caltag Laboratories, South San Francisco, CA; anti–Gr-1, RB6-8C5, PharMingen; TER119: erythroid lineage marker; kindly provided by Dr. T. Kina, Kyoto University) in PBS-BSA-EDTA. After 30 min of incubation on ice, the cells were washed twice with PBS-BSA-EDTA. To collect lineage marker-positive cells, the cells were sorted by FACStar PLUS (Becton Dickinson). Intraperitoneal macrophages, used as a source of mature macrophages, were obtained from 8 to 10-week old C57BL/6 mice.

Induction of the Differentiation of M1 Cells to Macrophages—The M1 Cell line clone T22–3 was obtained from Professor K. Nagata of Kyoto University. M1 cells were cultured in DME (Sigma) with 10% heat-inactivated horse serum. Before induction, M1 cells were morphological myeloblasts, but when incubated for 1–2 days with 50 units/ml of recombinant LIF (leukemia inhibitory factor) (ESGRO™ GIBCOBRL), they differentiated to macrophage-like cells and adhered to the substratum (*25*). Nonadherent cells were removed by pipetting and the remaining adherent cells were recovered by trypsin digestion.

Detection of MysPDZ Proteins by Western Blotting—The preparation of anti-MysPDZ was described previously (*1*). For Western blotting analysis, the cells were rinsed with PBS and harvested by scraping in cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150mM NaCl, 0.5% deoxycholate, 1% NP-40, 0.1% sodium azide) containing a protease inhibitor cocktail (BOEHRINGER Mannheim, Germany). Protein samples (30 μg/lane) were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), after which they were blotted onto PVDF membranes (Immobilon-P, MILLIPORE). The filters were immunoblotted with anti-MysPDZ antibody diluted at 40 ng/ml, and the bound antibodies were visualized with an ECL Plus Western blotting detection system (Amersham Pharmacia Biotech).

Subcellular Localization of MysPDZα and MysPDZβ—To prepare the MysPDZ-YFP fusion proteins, DNA fragments of MysPDZα and MysPDZβ were generated by PCR using KOD DNA polymerase (TOYOBO), and inserted into the Bgl II/Hind III sites of pEYFP-N1 (CLONTECH). A GFP-tagged mouse galactosyltransferase vector (GT-GFP) was kindly provided by Dr. M. Murata (National Institute of Physiological Science). The plasmids were transiently transfected into NIH3T3 by LipofectAMINE PLUS (Life Technologies) according to the manufacturer's instructions.

For immunofluorescence studies, cells were cultured on poly-L-lysine coated cover slips, fixed for 10 min at room temperature with 4% paraformaldehyde in PBS, permeabilized for 5 min with 0.1% Triton X-100 followed by washing with PBS, and then blocked for 30 min at 37°C with 10% FBS-PBS. After blocking, the cells were incubated for 2 h at room temperature with anti-MysPDZ antibody (4 μg/ml) diluted with blocking reagent. Then, the cells were washed and incubated with a labeled secondary antibody (Alexa Flour 488 goat anti-rabbit IgG, Molecular Probes, Cy3-conjugated AffiniPure donkey anti-rabbit IgG, Jackson Immuno Research). For staining of filamentous actin, the cells were incubated with rhodamine-phalloidin (5unit/ml, Molecular Probes).

RESULTS

Analysis of Variants of MysPDZ mRNA in Mouse Tissues—MysPDZ consists of a KE-rich sequence, PDZ domain, and the myosin homology region containing head, neck and tail regions typical of myosin family proteins (Fig. 1A). We previously showed by Northern blot analysis of MysPDZ that bone marrow contains two forms (7.5 kb and 7.0 kb) and that stromal cells express the 7.5 kb mRNA while the established hematopoietic cell lines express only the 7.0 kb mRNA (*1*). To confirm the different mRNA forms in hematopoietic (7.0 kb) and stromal cells (7.5 kb), Northern blot hybridization of mRNAs from bone marrow (7.0 kb, 7.5 kb), skeletal muscle (10.5 kb) and small intestine (7.5 kb) was performed with 3 different probes containing DNA sequences of 1–1230 nt (ORF containing the N-terminal PDZ-domain), 1231–3280 nt (ORF containing the head domain), and 3794–6108 nt (ORF of the neck and tail domains in the C-terminus) (Fig. 1A). In bone marrow, the probes containing the 1231–3280, and 3794–6108 ORFs hybridized with 7.5 kb MysPDZ mRNAs as detected in small intestine, the probe containing the 1–1230 ORF hybridized with the 7.5 kb, but not with the 7.0 kb mRNA. These results suggest that the 7.0 kb hematopoietic-specific form may lack the 5′ terminal sequence containing the ORF of the PDZ-domain, and these two forms may be generated by differential splicing. The stromal splicing isoform was

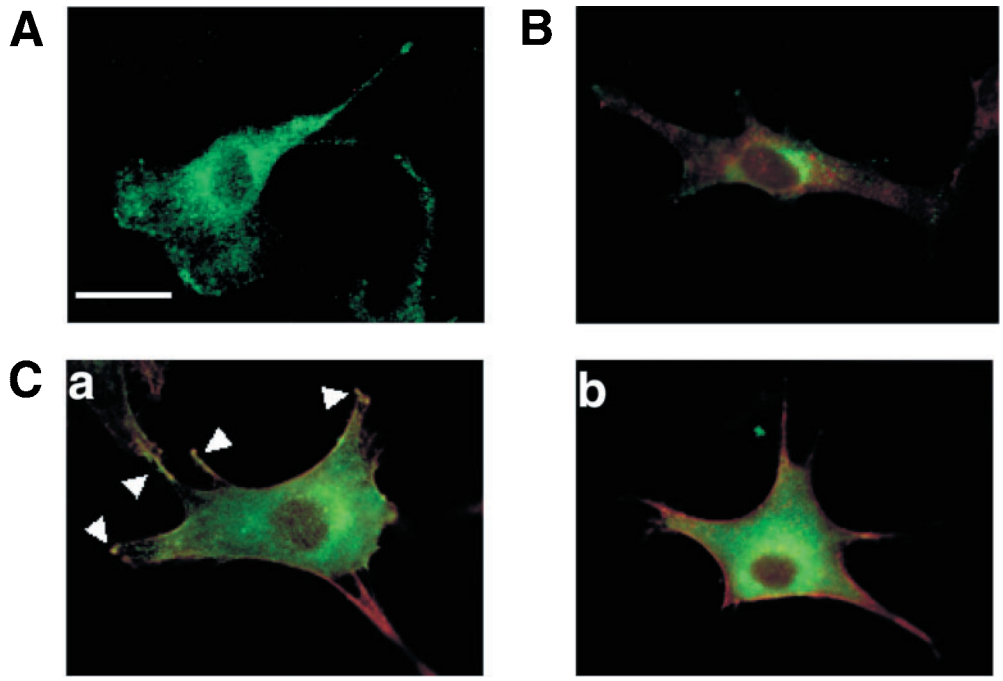


Fig. 4. **Subcellular localization of MysPDZα and MysPDZβ. Localization of MysPDZa in bone marrow stromal cells (TBR311).** MysPDZα was detected by indirect imunofluorescence using an affinity purified polyclonal antibody. MysPDZα is enriched in the juxtanuclear area and in the leading edge, and in the tip of filopodia. Co-localization of MysPDZα with the ER-Goldi complex. TBR311 cells transfected with GT-GFP were immunostained with anti-MysPDZ antibody. MysPDZα (red) was enriched in the juxtanuclear area, and co-localized with GT-GFP (yellow) on the ER-Golgi complex. Subcellular localization of MysPDZα or MysPDZβ. NIH3T3 cells transfected with MysPDZα-YFP or MysPDZβ-YFP were stained with rhodamine-labeled phalloidin to detect actin. (a) MysPDZα-YFP (green) showed a similar localization to endogenous MysPDZα in Fig. 4A, and partially co-localized (yellow) with actin (red) in the filopodia and peri-cellular region (arrowhead). (b) MysPDZβ-YFP (green) localized in the juxtanuclear area, but localized more diffusely in the cytoplasm than MysPDZα-YFP, and did not co-localize with actin in the filopodia or peri-cellular region. Bar indicates 20 μm.

called MysPDZα and the hematopoietic cell–specific isoform was called MysPDZβ.

Isolation of the cDNA for the MysPDZβ-Specific Spliced Isoform and Genome Structure of MysPDZ—To isolate the MysPDZβ-specific 5′-sequence, its cDNA was cloned by PCR amplification using a mouse spleen cDNA library made by λZIPLox phage DNA (see Fig. 2A). The cloned sequence contained 106 nt extending from the terminus of the myosin homologous region with the same ORF as that of MysPDZα, and this newly determined sequence lacked KE-rich- and PDZ-domains, completely differing from the 5′-terminus of the MysPDZα (Fig. 2B). To determine the 5′-terminus of the MysPDZβ mRNA, we surveyed the possible extended sequences of the MysPDZβ mRNA expecting a longer ORF from the GenBank database in human and mouse, and different potential primers were used to detect the 5′-extended sequence, however, none has yet been detected. Thus, the MysPDZβ-specific 5′-sequence containing the 106 nt extending from the terminus of the myosin homologous region may be the 5′-terminus of this spliced isoform. Thus MysPDZβ may lack the KE-rich– and PDZ domains. The generation of alternative spliced isoforms (MysPDZα and β) is described in Fig. 2C. Based on the sequence information present in the EMBL/GenBank/DDBJ database (EMBL/GenBank/DDBJ Acc. AL591065) and identified sequences (EMBL/GenBank/DDBJ Acc. AL591065) of the two spliced variants, we predicted the overall structure of the mouse MysPDZ gene (Fig. 2C); this gene consists of 40 exons.

Expression of Two Isoforms, MysPDZα and MysPDZβ, in Hematopoietic Cell Lines—The primer within the MysPDZβ 5′-specific sequence and the primer within the myosin homology region for MysPDZα were used to detect mRNA expression. When the expression of these two isoforms of MysPDZ mRNA was examined in mouse tissues, MysPDZα mRNA was detected in most tissues, whereas MysPDZβ mRNA was abundant in hematopoietic organs such as bone marrow, spleen and thymus (data not shown). Then, the expression of the two spliced isoforms was examined in several murine hematopoietic cell lines (Fig. 3A). MysPDZβ mRNA was detected in all hematopoietic cell lines and bone marrow. Some hematopoietic cell lines (J774.1, M1, NS-1 and M12) showed the expression of MysPDZα mRNA. The expression of the MysPDZ isoform proteins was confirmed by Western blot analysis using an anti-MysPDZ antibody (Fig. 3B). MysPDZα was detected as a 230 kD protein in the 3T3 fibroblast cell line analyzed by SDS–PAGE as previously reported (*1*). In 70Z/3 and EL-4, which express MysPDZβ mRNA but not MysPDZα mRNA, protein bands with a molecular size of 190 kDa were most abundant, while the 230 kDa protein was not detected. In contrast, J774.1 which expresses the mRNAs of isoforms, showed the 230 kDa band in addition to the 190 kDa band. M1 also showed the 230 kD aband weakly. Therefore, the MysP-

DZα mRNA codes for a 230 kDa protein and the MysPDZβ mRNA codes for a 190 kDa protein. The expression of both isoforms was confirmed at both the mRNA and protein levels in these hematopoietic cells.

Subcellular Localization of Two Isoforms, MysPDZα and MysPDZβ—To know the subcellular localization of the two isoforms of MysPDZ, polyclonal antibodies were raised to bacterially expressed fragments of MysPDZ. A highly specific antibody for MysPDZ was affinity-purified and used to study the localization of the protein by immunofluorescence in the bone marrow stromal cell line TBR311, which expresses the MysPDZα isoform abundantly, but not the MysPDZβ isoform.

The MysPDZ protein was visualized as distinct perinuclear dots that may co-localize with the ER-Golgi complex. It is also present in membrane ruffles and filopodia protruding from the plasma membrane, as well as being present in the cytosolic pool (Fig. 4A). To confirm the association of MysPDZ with the ER-Golgi complex, GFP-tagged mouse galactosyltransferase vector (GT-GFP) was transfected into TBR311 cells. The results showed clearly that both MysPDZ and GT-GFP co-localize on the ER-Golgi complex (Fig. 4B). These results suggest a role for MysPDZ in membrane traffic on secretory and endocytic pathways. Since MysPDZα contains the PDZ domain whereas MysPDZβ does not, it is interesting to know whether these two isoforms differ in their subcellular localization. When the hematopoietic cells (M1, 70Z/3, and EL-4 cells) that express only MysPDZβ were stained with the antibody, MysPDZ proteins were localized within the cytoplasm; however, their precise localization was not well visualized because of poor resolution (data not shown). Since the antibodies could not selectively detect the two isoforms, MysPDZ α and β were fused with YFP and transfected into NIH3T3 cells to detect whether the isoforms differ in their subcellular localization. MysPDZα-YFP containing the PDZ domain seemed to co-localize with both the ER-Golgi complex and the leading, ruffling edge of the cell (Fig. 4C-a), similar to the results of immunofluorescent staining shown in Fig. 4A. On the other hand, MysPDZβ-YFP lacking the PDZ domain seemed to localized diffusely within the cytoplasm and not to associate with the Golgi complex (Fig. 4C-b). In addition, when co-localization with actin was examined, MysPDZα-YFP was observed to co-localize partially with actin in the leading, ruffling edge of the cell (Fig. 4C-a), while MysPDZβ-YFP did not (Fig. 4C-b). These results suggest that the isoforms differ in their subcellular localization and may have different functions in membrane trafficking on secretory and endocytic pathways.

Expression of Isoforms in Normal Hematopoietic Progenitor Cells—To examine the expression of these isoforms in normal hematopoietic cells, different progenitor cells from bone marrow were fractionated by a cell sorter using monoclonal antibodies. Granulocytes (Gr-1$^+$), monocytes/macrophages (Mac-1$^+$), erythroid cells (Ter119$^+$) and lymphocytes (B220$^+$) were fractionated and the expression of the two isoforms was examined by the RT-PCR method (Fig. 5A). MysPDZβ mRNA was found to be expressed in granulocytes, monocytes/macrophages and lymphocytes, but not in erythroid cells. The absence of expression in erythroid cells was confirmed by titration

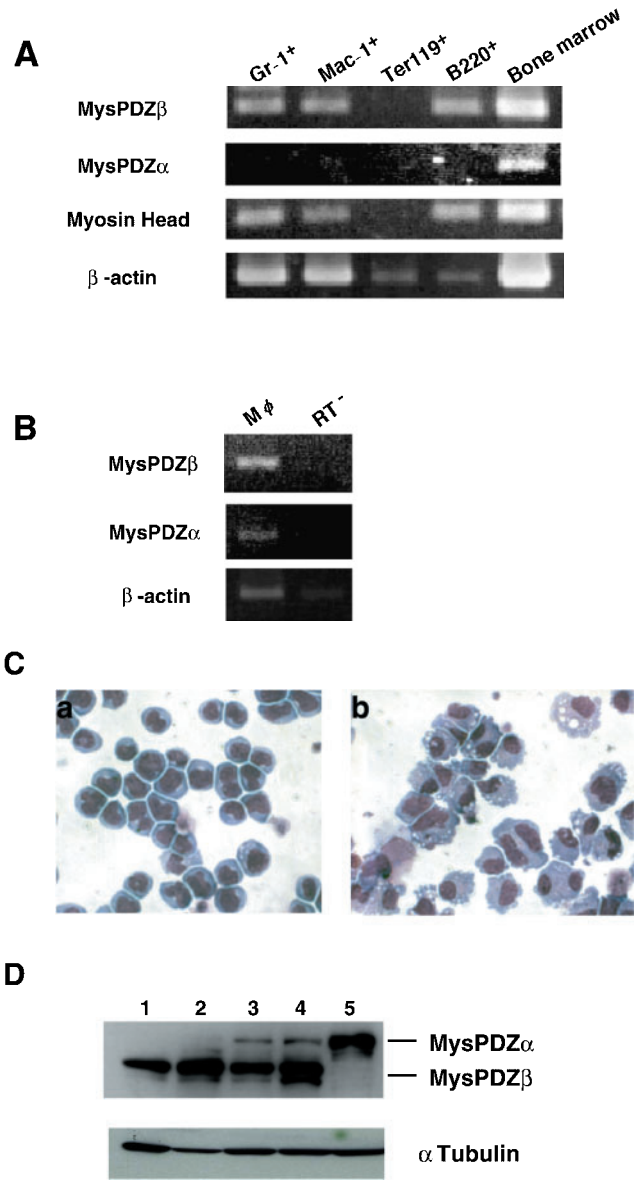


Fig. 5. **Expression of isoforms in normal hematopoietic cells and mature macrophages.** (A) The expression of MysPDZ isoform mRNAs in normal hematopoietic cells. Different lineages of hematopoietic cells in bone marrow were fractionated by a cell sorter using monoclonal antibodies. Granulocytes (Gr-1$^+$), monocyte/macrophages (Mac-1$^+$), erythroid cells (Ter119$^+$), and lymphocytes (B220$^+$) were fractioned and the expression of the two isoforms was examined by an RT-PCR method. MysPDZβ mRNA was detected in Gr-1$^+$, Mac-1$^+$, B220$^+$ cells, but not in Ter119$^+$ cells. In Ter119$^+$ cells, neither MysPDZβ mRNA nor MysPDZα mRNA was detected, and the absence of MysPDZ mRNA expression in erythroid cells was confirmed by titration with myosin head probes. (B) The expression of MysPDZ isoform mRNAs in mature macrophages. Intraperitoneal macrophages (Mø) were used as a source of mature macrophages. These cells expressed the mRNAs of both isoforms. (C) In vitro differentiation of M1 cells to macrophages. M1 cells could differentiate into macrophages in the presence of leukemia inhibitory factor (LIF). M1 cells without treatment (a), M1 cells treated with recombinant mouse LIF (rmLIF) for 48 h (b). (D) Expression of MysPDZα was induced by LIF treatment. Lane 1; preB cell line, 70Z/3, Lane 2; M1 cells without treatment, Lane 3; M1 cells treated with rmLIF for 24 h, Lane 4; M1 cells treated with rmLIF for 48 h, Lane 5; mouse bone marrow stromal cell line, TBR311.

with a myosin head probe. In contrast to the hematopoietic cell lines, none of the fractionated hematopoietic progenitor cells expressed MysPDZα mRNA at all. Next, we asked whether mature macrophages with strong adhesive function express MysPDZα mRNA. The results clearly showed that peritoneal macrophages as sources of mature macrophages express MysPDZα mRNA in addition to MysPDZβ mRNA (Fig. 5B).

Since mature macrophages express MysPDZα mRNA while immature macrophage progenitor cells (Mac-1$^+$ progenitors) do not, we asked whether MysPDZα expression is induced during the differentiation and maturation of macrophages. Mouse myeloid leukemia (M1) cells were induced to differentiate into macrophages by exposure to a differentiation-inducing factor, leukemia inhibitory factor (LIF) (*25*). When incubated for 1–2 days with LIF, M1 cells differentiated into macrophage-like cells and acquired motility and phagocytic activity, accompanied by the induction of adherence to the substratum (Fig. 5C). M1 cells expressed MysPDZβ abundantly before induction, and its level after induction was further elevated (Fig. 5D). Before induction, the MysPDZα protein was scarcely detectable, but significant amounts were observed after induction. When the adherent cells were separated from the nonadherent cells after induction, the former showed more abundant MysPDZα protein than the latter. These results indicate that MysPDZα is induced depending on the differentiation of macrophages.

DISCUSSION

We previously cloned a gene for a novel myosin (Myosin containing PDZ domain = MysPDZ) from bone marrow stromal cells (*1*). This myosin has an N-terminal PDZ domain, one IQ motif, and a tail of segmented coiled-coil. In the present study, we identified a new spliced isoform (MysPDZβ), which lacks the PDZ domain and is expressed specifically in murine hematopoietic cells. MysPDZβ is expressed in most hematopoietic cell lines and fractionated bone marrow hematopoietic cell fractions, whereas stromal cells and many other tissues express MysPDZα but not MysPDZβ, suggesting differing functional roles of these isoforms.

This unconventional myosin was classified as class XVIII, and the human MysPDZ gene was named MYO18A in a recent review (*11*). We found a human homologue in the GenBank database that is 94% identical overall but lacks the N-terminal PDZ domain (KIAA0216, Acc. no. D86970). Since the KIAA0216 sequences were cloned from the cDNA of a human hematopoietic cell line, it is likely that KIAA0216, which lacks the N-terminal PDZ domain, may be the human spliced isoform (hMysPDZβ) expressed in hematopoietic cells. In addition, a single exon from chromosome 17 BAC (Acc. no. AC005412) encodes a peptide of ~350-aa that matches with ~94% identity the N-terminal PDZ domain of mouse MysPDZ; this was found in the GenBank database and may be the human spliced isoform (hMysPDZα) expressed in stromal cells. Thus, overall, the spliced isoforms are expressed similarly in human and mouse tissues.

Part of a gene somewhat similar to the human PDZ-myosin was also identified by genome annotators, on a BAC from human chromosome 22. The full-length predicted protein shares ~40% protein identity with human and mouse MysPDZ and groups reliably with other class XVIII myosins in phylogenetic trees, suggesting that the gene should be termed MYO18B. Despite the general similarity to PDZ-myosins, exons coding for a PDZ sequence were not identified in the genomic sequence upstream of the head domain in MYO18B (*11*).

Based on the sequence information present in the GenBank database and the identified sequences of the two spliced isoforms, we predicted the overall structure of the mouse MysPDZ gene (Myo18A) (Fig. 2C). This gene consists of 40 exons and generates at least two spliced isoforms. Since the spliced forms other than MysPDZ α and β might be expressed in brain, heart, skeletal muscle and even in stromal cells based on the results of Northern blot hybridization, MysPDZ may generate different forms of protein that contribute to a variety of functions in different tissues, similar to several classes of unconventional myosins that have alternative splicing isoforms (*13–22*). Yamashita *et al.* (*12*) found the predicted Drosophila myosin gene at 89B (CG10218, Acc. no. AAF55271 and AAF55272), and, recently, Tzolovsky *et al.* reported the presence of class XVIII PDZ-myosins in Drosophila (*13*). Given the recent discovery of PDZ-containing myosins in vertebrates, the presence of this domain and phylogenetic analysis clearly indicate a relationship between the fly and vertebrate class XVIII myosins.

Although the function of this novel myosin is not yet known, the identification of two spliced isoforms of MysPDZ with and without the PDZ domain is intriguing, because this domain may function as part of a mobile scaffolding complex through protein-protein interaction. Immunofluorescence studies showed that MysPDZα containing the PDZ domain seems to co-localize with the ER-Golgi complex, while MysPDZβ lacking the PDZ domain seems not to co-localize with the ER-Golgi complex. Taken together with their co-localization with actin, both isoforms were shown to differ in their subcellular localization, and, thus, they may have different functions in membrane ruffling and membrane trafficking pathways (*30–32*).

Whereas the MysPDZα mRNA is expressed ubiquitously in many tissues, the MysPDZβ mRNA is expressed specifically in hematopoietic cells. Among fractionated hematopoietic cells from bone marrow, MysPDZβ mRNA is expressed in granulocytes, monocytes/macrophages and lymphocytes, but not in erythroid cells. The complete lack of expression of MysPDZ in erythroid cells may be related to the unique membranous structure of red blood cells and the shape stabilization of the red blood cell membrane. Few PDZ-containing proteins have been reported in hematopoietic cells. Human p55, the major palmitoylated protein associated with the cytoplasmic face of the erythrocyte membrane, is believed to modulate interactions between protein 4.1 and glycophorin C (*23*, *24*). RT-PCR of the p55 mRNA from a patient with acute megakaryoblastic CML revealed a 69 base pair deletion in the PDZ domain, corresponding to exon 5 of the p55 gene. The deletion of constitutive exon 5 not only marks the first p55 cDNA abnormality in human disease, but also the first PDZ domain abnormality in human dis-

ease, and may represent another genetic abnormality associated with CML in blast crisis.

While most hematopoietic progenitor cells do not express the MysPDZα mRNA at all, some hematopoietic cell lines (J774.1, M1, NS-1, and M12) show expression. The MysPDZα expressing hematopoietic cell lines seem to adhere to the substratum during culture, whereas the non-expressing cells do not. We found that peritoneal macrophages express MysPDZα mRNA in addition to MysPDZβ mRNA (Fig. 5B), although immature macrophage progenitor cells (Mac-1+ progenitors) in bone marrow do not. Furthermore, MysPDZα was found to be induced depending on the differentiation and maturation of myeloid leukemia (M1) cells cultured in the presence of LIF. Actin-based motor protein requirements are important features of macrophage activity during phagocytosis or microbicidal processes. Different classes of myosins have been shown to contribute directly or indirectly to phagocytosis by providing the mechanical force for phagosome closure or organelle movement (*26*, *27*). Reis *et al.* (*28*) reported the presence of different classes of myosins (Myosins IC, II, Va, VI and IXb) in J774 macrophages and showed that there is an increase in myosin V expression as well as iNOS expression following treatment with IFNγ, which is involved in macrophage activation. Wirth *et al.* showed that the expression of human myosin IXb increases when a human myelocytic cell line, HL-60, is induced to differentiate into macrophage-like cells (*29*), and Cox *et al*. (*30*) showed that myosin X is a downstream effector of PI(3)K during phagocytosis. Thus, the presence of PDZ-containing MysPDZ (MysPDZα) seems to be related to the adherence and/or some other functions of mature macrophages.

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REFERENCES

1. Furusawa, T., Ikawa, S., Yanai, N., and Obinata, M. (2000) Isolation of a novel PDZ-containing myosin from hematopoietic supportive bone marrow stromal cell lines. *Biophys. Biochem. Res. Commun.* **270**, 67–75
2. Mermal, V., Post, P.L., and Mooseker, M.S. (1998) Unconventional myosins in cell movement, membrane traffic, and signal transduction. *Science.* **279**, 527–533
3. Sellers, J.R. (2000) Myosins: a diverse superfamily. *Biochim Biophys Acta*. **12**, 42–51
4. Tuxworth, R.I. and Titus, M.A. (2000) Unconventional myosins: anchors in the membrane traffic relay. *Traffic.* **1**, 11–18
5. Wu, X., Jung, G., and Hammer, J.A.III. (2000) Functions of unconventional myosins. *Curr. Opin. Cell. Biol.* **12**, 42–51
6. Kornau, H.C., Schenker, L.T., Kennedy, M.B., and Seeburg, P.H. (1995) *Science*. **269**, 1737–1740
7. Niethammer, M., Valtschanoff, J.G., Kapoor, T.M., Allison, D.W., Weinberg, T.M., Craig, A.M., and Sheng, M. (1998) CRIPT, a novel postsynaptic protein that binds to the third PDZ domain of PSD-95/SAP90. *Neuron*. **20**, 693–707
8. Brenman, J.E., Topinka, J.R., Cooper, E.C., McGee, A.W., Rosen, J., Milroy, T., Ralston, H.J., and Bredt, D.S. (1998) Localization of postsynaptic density-93 to dendritic microtubules and interaction with microtubule-associated protein 1A. *J. Neurosci.* **18**, 8805–8813
9. Hildebrand, J.D. and Soriano, P. (1999) Shroom, a PDZ domain-containing actin-binding protein, is required for neural tube morphogenesis in mice. *Cell*. **99**, 485–497
10. Furuyashiki, T., Fujisawa, K., Fujita, A., Madaule, P., Uchino, S., Mishina, M., Bito, H., and Narumiya, S. (1999) Citron, a Rho-target, interacts with PSD-95/SAP-90 at glutamatergic synapses in the thalamus. *J. Neurosci.* **19**, 109–118
11. Berg, J.S., Powell, B.C., and Cheney, R.E. (2001) A millennial myosin census. *Mol. Biol. Cell.* **12**, 780–794
12. Yamashita, R.A., Sellers, J.R., and Anderson, J.B. (2000) Identification and analysis of the myosin superfamily in Drosophila: a database approach. *J. Muscle Res. Cell. Motil.* **21**, 491–505
13. Tzolovsky, G., Millo, H., Pathirana, S., Wood, T., and Bownes, M. (2002) Identification and phylogenetic analysis of Drosophila melanogaster myosins. *Mol. Biol. Evol.* **19**, 1041–1052
14. Lambert, J., Naeyaert, J.M., Callens, T., De Paepe, A., and Messiaen, L. (1998) Human myosin V gene produces different transcripts in a cell type-specific manner. *Biochem. Biophys. Res. Commun.* **252**, 329–333
15. Wu, X., Wang, F., Rao, K., Sellers, J.R., and Hammer, J.A.3rd. (2002) Rab27a is an essential component of melanosome receptor for myosin. *Mol. Biol. Cell*. **13**, 1735–1749
16. Chen, A., Wayne, S., Bell, A., Ramesh, A., Srisailapathy, C.R.S., Scott, D.A., Sheffield, V.C., van Hauwe, P., Zbar, R.I.S., Ashley, J., Lovett, M., van Camp, G., and Smith, R.J.H. (1997) New gene for autosomal recessive non-syndromic hearing loss maps to either chromosome 3q or 19p. *Am. J. Med. Genet.* **71**, 467–471
17. Kelley, P.M., Weston, M.D., Chen, Z.Y., Orten, D.J., Hasson, T., Overbeck, L.D., Pinnt, J., Talmadge, C.B., Ing, P., Mooseker, M.S., Corey, D., Sumegi, J., and Kimberling, W.J. (1997) The genomic structure of the gene defective in Usher syndrome type Ib (MYO7A). *Genomics* **40**, 73–79
18. Mburu, P., Liu, X.Z., Walsh, J., Saw, D., Cope, M.J.T.V., Gibson, F., Kendrick-Jones, J., Steel, K.P., and Brown, S.D.M. (1997) Mutation analysis of the mouse myosin VIIA deafness gene. *Genes Funct.* **1**, 191–203
19. Chieregatti, E., Gärtner, A., Stöfler, H.-E., and Bähler, M. (1998). Myr 7 is a novel myosin IX-RhoGAP expressed in rat brain.*J. Cell Sci.* **111**, 3597–3608
20. Grewal, P.K., Jones, A.M., Maconochie, M., Lemmers, R.J., Frants, R.R., and Hewitt, J.E. (1999) Cloning of the murine unconventional myosin gene Myo9b and identification of alternative splicing. *Gene* **240**, 389–398
21. Hasson, T. and Mooseker, T.M. (1996) Vertebrate unconventional myosins. *J.Biol.Chem*. **271**, 16431–16434
22. Pestic-Dragovich, L., Stojiljkovic, L.P., and de Lanerolle, P. (2000) A myosin I isoform in the nucleus. *Science* **290**, 337–341
23. Marfatia, S.M., Morais-Cabral, J.H., Kim, A.C., Byron, O., and Chishti, A.H. (1997) The PDZ domain of human erythrocyte p55 mediates its binding to the cytoplasmic carboxyl terminus of glycophorin C. Analysis of the binding interface by in vitro mutagenesis. *J. Biol. Chem.* **272**, 24191–2497
24. Ruff, P., Chishti, A.H., Grimm, E., Bischoff, D., and Kim, A.C. (1999) Exon skipping truncates the PDZ domain of human erythroid p55 in a patient with chronic myeloid leukemia in acute megakaryoblastic blast crisis. *Leuk. Res.* **23**, 247–250
25. Tsuru, A., Nakamura, N., Takayama, E., Suzuki, Y., Hirayoshi, K., and Nagata, K. (1990) Regulation of the expression of vimentin gene during the differentiation of mouse myeloid leukemia cells. *J. Cell Biol.* **110**, 1655–1664
26. Swanson, J.A., Johnson, M.T., Beningo, K., Post, P., Mooseker, M., and Araki, N. (1999) A contractile activity that closes phagosomes in macrophages. *J. Cell Sci.* **112**, 307–316
27. Defacque, H., Egeberg, M., Habermann, A., Diakonova, M., Roy, C., Mangeat, P., Voelter, W., Marriott, G., Pfannstiel, J., Faulstich, H., and Griffiths, G. (2000) Involvement of ezrin/moesin in de novo actin assembly on phagosomal membranes. *EMBO J.* **19**, 199–212
28. Reis, D., Souza, M., Mineo, J., and Espindola, F. (2001). Myosin V and iNOS expression is enhanced in J774 murine macro-

phages treated with IFN-gamma. *Braz. J. Med. Biol. Res.* **34**, 221–226
29. Wirth, J.A., Jensen, K.A., Post, P.L., Bement, W.M., and Mooseker, M.S. (1996) Human myosin-IXb, an unconventional myosin with a chimerin-like rho/rac GTPase-activating protein domain in its tail. *J.Cell. Sci*. **109**, 653–661
30. Cox, D., Berg, J.S., Cammer, M., Chinegwundoh, J.O., Dale, B.M., Cheney, R.E., and Greenberg, S. (2002) Myosin X is a downstream effector of PI(3)K during phagocytosis. *Nat. Cell. Biol*. **4**, 469–477
31. Wu, X., Wang, F., Rao, K., Zhang, H., Wang, F., Sellers, J.R., Matesic, L.E., Copeland, N.G., Jenkins, N.A., and Hammer, J.A.III. (2002) Identification of an organelle receptor for myosin-Va. *Nat. Cell. Biol.* **4**, 271–278
32. Buss, F., Kendrick-Jones, J., Lionne, C., Knight, A.E., Cote, G.P., and Luzio, J.P. (1998) The Localization of Myosin VI at the Golgi Complex and Leading Edge of Fibroblasts and Its Phosphorylation and Recruitment into Membrane Ruffles of A431 Cells after Growth Factor Stimulation. *J. Cell Biol.* **143**, 1535–1545