Mammalian Myosin Iα, Iβ, and Iγ: New Widely Expressed Genes of the Myosin I Family

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Abstract. A polymerase chain reaction strategy was devised to identify new members of the mammalian myosin I family of actin-based motors. Using cellular RNA from mouse granular neurons and PC12 cells, we have cloned and sequenced three 1.2-kb polymerase chain reaction products that correspond to novel mammalian myosin I genes designated MMIα, MMIB, MMIγ. The pattern of expression for each of the myosin I's is unique: messages are detected in diverse tissues including the brain, lung, kidney, liver, intestine, and adrenal gland. Overlapping clones representing full-length cDNAs for MMIα were obtained from mouse brain. These encode a 1,079 amino acid protein containing a myosin head, a domain with five calmodulin binding sites, and a positively charged COOH-terminal tail. In situ hybridization reveals that MMIα is highly expressed in virtually all neurons (but not glia) in the postnatal and adult mouse brain and in neuroblasts of the cerebellar external granular layer. Expression varies in different brain regions and undergoes developmental regulation. Myosin I's are present in diverse organisms from protozoa to vertebrates. This and the expression of three novel members of this family in brain and other mammalian tissues suggests that they may participate in critical and fundamental cellular processes.

The myosin I family of proteins, like conventional muscle myosins (myosin II), are actin-activated ATPases that generate mechanochemical force within the cell (Korn and Hammer, 1989; Pollard et al., 1991). Unlike the bipolar filaments of myosin II, myosin I's in solution exist as monomeric globular proteins. All myosin I's identified share two important structural properties: a myosin head that contains sites for ATP and actin binding, and a COOH-terminal domain that binds to membranes through interaction with negatively charged phospholipids (Adams and Pollard, 1989). Since the initial report of myosin I in Acanthamoeba (Pollard and Korn, 1973), the number and diversity of myosin I family members have grown. Although myosin I's have been well studied in organisms including Acanthamoeba (Jung et al., 1987; Lynch et al., 1989), Dictyostelium (Jung et al., 1989; Jung and Hammer, 1990; Titus et al., 1989), and Drosophila (Montell and Rubin, 1989; Hicks and Williams, 1992), only a single myosin I gene in vertebrates has been cloned and extensively characterized to date (Hoshimaru and Nakanishi, 1987; Garcia et al., 1989). This species appears to be localized exclusively to the brush border of the small intestine (Hoshimaru et al., 1989; Bikle et al., 1991) and has been designated brush border myosin I (BBMI).1 Recent biochemical findings have pointed to the presence of additional mammalian myosin I family members (Coluccio, 1991; Barylko et al., 1992).

Biochemical, immunolocalization, and EM studies have suggested that myosin I's play diverse and important roles within cells (for review see Korn and Hammer, 1990; Hammer, 1991; Pollard et al., 1991; Cheney and Mooseker, 1992). For example, in Dictyostelium, myosin I was localized to the leading edge of motile cells and to the phagocytic cup upon ingestion of bacteria (Fukui et al., 1989). Similarly, in Acanthamoeba, myosin IC was found both at the plasma membrane and the contractile vacuole (Pollard and Korn, 1990), and in chick intestinal microvilli, BBMI forms the crossbridge between the actin bundle core and the plasma membrane (Mooseker and Tilney, 1975). In addition, functional loss of the single myosin II gene in Dictyostelium did not cause defects in pseudopod extension, cell motility, phagocytosis, or retrograde movement of membrane antigens (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Jay and Elson, 1992), all actin-based activities involving interaction with the plasma membrane that could be directed by myosin I's.

We have approached our studies of myosin I's with an interest in their potential roles within the vertebrate nervous system. Cell migration, neurite outgrowth, and vesicular transport are all processes that might involve myosin I's (Mitchison and Kirschner, 1988; Smith, 1988; Pollard et al., 1991) and that are central to neuronal development. Myosin I-like proteins have been localized to growth cones by immunostaining of a neuronal cell line (Miller et al., 1992) and by immunoblotting of fractions enriched in growth cone membranes (Phelan et al., 1991). To begin to address the is-

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1. Abbreviations used in this paper: BBMI, brush border myosin I; EGL, external granular layer.
sue of the role of myosin I in the brain, we have devised and implemented a PCR-based strategy to clone and characterize novel myosin I genes expressed in the nervous system.

Portions of this work have been presented previously in abstract form (Sherr and Greene, 1991. J. Cell Biol. 115:33 Lz; and Joyce et al. 1992. Soc. Neurosci. Abstr. 18:221).

Materials and Methods

Generation of PCR Products

The degenerate primers used in this study and their corresponding conserved amino acid motifs were as follows: 5'-ATACTGAGAAGCTTCG-GWGGCIGGIAARCNAGGC-3' (GAGKTE); 5'-ATACAGAATCATCGGTTCG-TACGCGACGAC-3' (LEFNAGK); 5'-ATAAGATTATCAGGTTGTTCGTTTTATCCACCTATRA-3' (VIRCIKPN); and 5'-ATAGAATTATCGATACRYYYCTTCAGCAGNC-CAGRTA-3' (YLLLENV). The two 5' primers contained Xba I and HindIII restriction sites 5' of the degenerate nucleotides, while the 3' primers included the EcoRI and ClaI sites. The primary templates were total RNA from PC12 cells and P6 mouse cerebellar granular neurons. The secondary template was a 1-5 ml of unpurified material from the first amplification. The annealing temperature was 48°C in the first round and 54°C in the second. The remaining conditions were those recommended by the manufacturer (Perkin-Elmer Cetus, Norwalk, CT). Bovine BBM1 cDNA (Hoshimaru and Nakanishi, 1987), kindly provided by Dr. S. Nakanishi (Kyoto University, Kyoto, Japan), served as the positive control. PCR products of the predicted size were purified from agarose gels (Genclean, Bio 101, New York University, New York, NY) and directionally cloned into pGEMZf+ (Promega Corp., Madison, WI) at Xba I and EcoRI. Clones were analyzed by direct sequencing and by restriction digestion patterns.

RNA Transfer Analysis

Total RNA was purified from tissues and cell lines according to the procedure of Chomczynski and Sacchi (1987). RNA transfer was performed with standard protocols (Sambrook et al., 1989). Probes were labeled by random priming with [32P]dCTP as described above.

Cell Cultures and Isolation

P19 teratocarcinoma cells (McBurney and Rogers, 1982), N115 neuroblastoma cells (Schrier et al., 1974), U251 and G24-26 astrocytoma cells (Potten, 1975; Sundarrag et al., 1975), and C17 cells (Ryder et al., 1990) were grown under standard conditions and harvested directly for the isolation of total RNA. PC12 cells (Greene and Tscherter, 1976) were treated with NGF (100 ng/ml) for 5 d before harvesting. Astrocytes were purified from rat P6 cerebellum as described (Hatten, 1985).

Animals and Tissue Preparation

P0, P2, P7, P12, and adult mice were deeply anesthetized with an intraperitoneal injection of an overdose of ketamine. The mice were perfused transcardially with 0.1 M PBS, pH 7.2, prepared with diethyl pyrocarbonate-treated water. Paraffin-embedded sections were treated with proteinase K (20 µg/ml in 10 mM Tris, 1 mM EDTA (TE)) for 25 min at room temperature. The sections were rinsed in TE, washed in 2x SSC for 10 min, and prehybridized in buffer containing 50% formamide, 20x SSC, 2 M Tris, pH 7.5, 50x Denhardt's solution, and 0.1 mg/ml salmon sperm DNA for 1 h at room temperature. Sections were incubated with the digoxigenin-UTP-labeled probe (4 µg/ml prehybridization solution) for 16 h at 42°C. The next day, sections were briefly rinsed in 2x SSC and incubated in RNase A (200 µg/ml) in 10 mM Tris, 500 mM NaCl, and 1 mM EDTA for 15 min at 37°C. The sections were then washed with 2x SSC for 30 min at room temperature, twice in 0.5x SSC for 15 min at 42°C, once in 0.1x SSC for 1 h at room temperature, and briefly in 100 mM Tris and 150 mM NaCl, pH 7.5 (buffer 1). The sections were then incubated in 2% normal sheep serum with 0.3% Triton X-100 in Buffer I for 30 min at room temperature. The sections were then incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim Corp.) diluted 1:100 in buffer 1 with 2% normal sheep serum and 1.25% Triton X-100 overnight at 4°C. The next day the tissue was washed in buffer I three times for 10 min each, followed by a 2-min wash in 100 mM Tris, 100 mM NaCl, and 50 mM MgCl2, pH 9.5 (buffer III). The reaction product was visualized with 0.023 µM nitro blue tetrazolium chloride, 0.018% 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate), and 0.024% levamisole. The blue-brown color reaction product was visible within 1-2 h. The sections were then washed in buffer IV for at least 5 min to stop the reaction. Control conditions included omission of the digoxigenin-UTP-labeled probe, labeled sense RNA, and competition with unlabeled antisense RNA. Sections were coveredslipped in AquaMount, Lerner Laboratories (Pittsburgh, PA).

SI Nuclease Protection

Probes including 46 and 51 nucleotides (MM10: 5'-CAAGTAGCTCGGGGGTTCGTTCTCCAGAGCGACGAGACGAC-3', MM1: 5'-CACTTCAGGTTGCCCCACACGAGATGAGTTCAGGATGATT-3') corresponding to stretches within pGMMM1 and pGMMy, respectively, that are of low homology among currently described myosin I genes, were synthesized for hybridization to total tissue RNA. SI nuclease protection was carried out following a standard protocol as described (Greene and Struhl, 1987). Briefly, 2 nml of the oligonucleotide probes was end labeled with [32P]dCTP by polynucleotide kinase (Boehringer Mannheim Corp.). 1.2 x 106 cpm for each probe was hybridized with 25 µg of total RNA overnight at 30°C. 300 U of SI nuclease (Boehringer
Results

Identification of Novel Mammalian Myosin I Genes

Both preliminary biochemical studies (Phelan et al., 1991) and the presence of multiple myosin I's in lower organisms (Pollard et al., 1991) suggested the existence of a family of mammalian myosin I genes in addition to the well-characterized BBMIs. To search for such genes, we devised a two-step nested PCR strategy that was biased against amplification of conventional myosin II sequences. Two pairs of degenerate primers from conserved amino acid sequences within the myosin head domain (GAGKTEA and YLGLEN; LEAFGN and YIRCtIkP) were used in two sequential reactions. Because we sought to identify myosin I genes expressed in the nervous system, total cellular RNA from PC12 cells and postnatal day 6 mouse cerebellar granular neurons served as the templates for the first reaction. The second amplification was conducted with the nested primers using the unpurified products of the first reaction as the templates. 42 subcloned PCR products (30 from PC12 cells and 12 from granular neurons) were analyzed by restriction fragment patterns and partial sequencing. Three unique PCR products were identified and sequenced. Analysis of the nucleotide and predicted amino acid sequences confirmed that these fragments correspond to novel myosin I genes (Fig. 1A). The clone derived from mouse cerebellar granular neuron RNA, designated pGMMI~a, is 66 and 64% identical (based on the predicted amino acid sequence) to bovine and chicken BBMIs, respectively. Over the same stretch, pGMMI~a is 38% identical to cardiac muscle myosin. Of the conventional myosin sequences contained in Genbank, this myosin II gene is the most similar to pGMMI~a. Clones pGMMI~a and pGMMI~a, derived from PC12 cells, are 49% identical to bovine BBMIs, but only 37% homologous to the cardiac muscle myosin sequence. Additionally, there is a highly conserved stretch within all described myosin I head domains that distinguishes them from myosin II. Sequences similar to this motif, QA-yRDAI-ALAIYSR (Pollard et al., 1991), are present in all three clones. Clone pGMMI~a, which diverges the most from this motif, is still 73% identical in this stretch.

Cloning and Predicted Primary Sequence of Mammalian Myosin Iα

As will be detailed below, using clone pGMMI~a as a probe we detected a message with a distinctive pattern of regulation in the developing postnatal mouse brain and therefore chose this clone for further study. Screening an oligo-dT primed cDNA library from postnatal day 6 mouse cerebellar granular neurons yielded three overlapping clones ranging from 2.5 to 3.3 kb. Each of the three clones encodes a stretch of the cDNA from within the consensus myosin head through to the 3' untranslated sequence. Two of these clones, λCGN74a and λCGN82a (see Materials and Methods), while identical in the coding region, differ in the 3' untranslated stretch, with λCGN74a containing an additional 503 nucleotides before the polyadenylation site (Fig. 2).

To obtain a clone encoding the 5' end, we screened a random primed mouse brain library with a PCR product that extended 5' from the cDNA clone λCGN82a (see Materials and Methods). We isolated a 1.2-kb clone (λAMB9d) that overlapped with λCGN82a and extended 850 nucleotides to the 5' end. These overlapping clones contain a single long open reading frame that begins with a methionine codon at the designated nucleotide 1. This sequence ACCATG is predicted to be a strong translation initiation site (Kozak, 1989). The only in-frame methionine upstream, at position -17, would constitute a weak initiation codon. Translation of this open reading frame predicts a protein of 1,079 amino acids with a molecular mass of 125,160 D (Fig. 2).

Analysis of the predicted MMα protein reveals three functional domains that are similar to the BBMIs: a myosin head, a calmodulin binding region, and a COOH-terminal tail with a high net positive charge. All myosin heavy chain proteins share a head domain that contains nucleotide and actin binding sites as well as other conserved residues that are probably necessary for the generation of mechanochemical force (Korn and Hammer, 1988). Analysis reveals that MMα protein contains such a canonical myosin head (Fig. 2). A sequence that participates in ATP binding, GESGAKE, is conserved throughout all myosins and is also present in MMα (Fig. 2). Moreover, MMα mRNA encodes an actin binding site, YIRCtIkP(N/K), that is shared by myosin I proteins and is distinct from myosin II. These features provide further evidence that MMα encodes a member of the myosin I family.

Immediately COOH terminal to the head domain, the predicted MMα protein appears to encode, by comparison to BBMIs, a series of calmodulin binding sites. Calmodulin serves as the light chain for BBMIs and other unconventional vertebrate myosins (Mooskeker and Coleman, 1989; Esparis et al., 1992), and its interaction with Ca2+ has been shown to regulate ATPase activity and in vitro motility (Mooskee et al., 1989; Collins et al., 1990). This region was mapped in the chick BBMIs by α-chymotryptic mapping and construction of fusion proteins with calmodulin binding capacity (Coluccio and Bretscher, 1988; Halsall and Hammer, 1990). Comparison of this domain in BBMIs with the corresponding region in the MMα protein shows an amino acid identity of 56% and, with conservative substitutions, a sequence similarity of 74%. Prior reports evaluating this domain in BBMIs have based estimates of the number of sites on an alignment of this region with the well-defined calmodulin binding domain of neuromodulin (Swanljung-Collins and Collins, 1992), and with a similar motif in neurogranin (Baudier et al., 1991). This comparison was based on the observation that both BBMIs and neuromodulin bind calmodulin in the absence of Ca2+ (Coluccio and Bretscher, 1987; Alexander et al., 1988). A similar comparison of this region in MMα reveals five potential calmodulin binding sites (Fig. 2). There are conserved aromatic or aliphatic residues at positions 1, 5, and 8, as well as basic amino acids clustered in the COOH-terminal part of these sites.

The alignment of the calmodulin binding region also reveals a 29 amino acid insert within MMα that is absent in bovine BBMIs. An insert, similar in both length and relative position within the message, was recently reported as a rare splice variant of chicken BBMIs (Fig. 2) (Halsall and Hammer, 1990). A synthetic peptide of this fragment was shown...
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that are highly conserved in the myosin I but not in the myosin II gene family are underlined. Where applicable, numbers indicate the amino acid residue according to published sequences. The residue numbers for MMI/3 and MMI,y start at the 5' ends of the PCR products. The sequences are not listed in the order of their relatedness. MMIv does not extend to the 3' protein sequences were aligned within the overlapping head domain using the Pileup program (Devereux et al., 1984) of the GCG package.

A Consensus

ChkBBMI GDVAVTVVES AKGGGDVPVC
BovBBMI GSLTVKVIQG PGGGGTGKLS

BovBBMI QAKTVIPLNS LAGVSVTSFK
BovBBMI ASELFKGKKA SYPQSVPIPF

ChkBBMI PGKQRKVMGV LDIYGFEIFQ
ChkBBMI E.ISQLIGLD PSTLEQALCS

ChkBBMI LEAFGNAKTI RNDNSSRFGK
ChkBBMI NNDLLFRDLS QAMWAARHTL

MMI~ DKVCVKFIQG NQKNGSVPTC
MMI~ QIKSEVPLVD VTKVSMSSQN

MMI~ .SELFKDKKA LYSVVQGQF QQAYLEIKNN PKYKLKDAD EEEIIIABV NKNIRANGKS TSFPLFILLPN NLLLADQKGG 65
BovBBMI ASELFKDKKA SYQQSVQPFP HGVDYIQGNR PNLKQLGGKE EGPILAVEBTV KVHKNKAGY SSRLILLKFG HIIVDTMKPN 54
ChkBBMI .QLSFQKDKA LYSQQVQGF QEGYCHGFQN RKYCLQAVA KELDVMALN QVKNRANGKT TSFPLLTLPT HVLADPKFA 112
Consensus .SELFKDKKA 1YP-sv.q.PP .G.YI-N ... p.KR-E .. P .TAGQPKFSV .ATLMNQLTQ NPNYIRCIP 599

MIa LEAPGAKTV RNDSSRFPK YMDVPDEFPK DPLLQVSNY LLEKVSVQPK PHGGRNHPVF YQLLGSASG LLYLLKLERD 228
MIb LEAPGAKVL RNINSPRPK YMDVPDEFPK APVGCHLSY LLEKVSVQPK NQGERNPVFV YQLLGGSEE LSLALIERNR 80
MIy LEAPGAKVTL RNINSPRPK YMDVPDEFPK APVGCHLSY LLEKVSVQPK QPQGRSHSF YQLLQOSQG MIASLHQLK 80
BoVBMMI LEAPGAKTV RNINSPRPK YMDVPDEFPK DPLLQVSNY LLEKVSVQPK TGERNHPVF YQLLAGDAQ LLKALLLERD 221
ChkBMII LEAPGAKTV RNNSSSRFPK YFEFHPKRLK EPCGRITNIV LLEKVSVQPK QPQGRSHSF YQLLQOSQG MIASLHQLK 80
AacBIb LEAPGAKTV RNNSSSRFPK YFEFHPKRLK PECPGRITNIV LLEKVSVQPK QPQGRSHSF YQLLAGASA EAEQMLgly.A 112
CrdMIi LEAPGAKTV RNNSSSRFPK YFEFHPKRLK EPCGRITNIV LLEKVSVQPK QPQGRSHSF YQLLAGASA EAEQMLgly.A 112

Figure 1. Alignment of myosin I head and tail sequences. (A) Predicted protein sequences for MI, MI\beta, MI\gamma, and other myosin protein sequences were aligned within the overlapping head domain using the Pileup program (Devereux et al., 1984) of the GCG package. Where applicable, numbers indicate the amino acid residue according to published sequences. The residue numbers for MI\gamma and MI\gamma do not extend to the 3' end because this PCR product was cut at a HindIII site at serine 427 in preparation for subcloning. Examples of residue domains that are highly conserved in the myosin I but not in the MI\gamma gene family are underlined. BoVBMMI, bovine BBMI (Hoshimarud

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to bind calmodulin, also independent of Ca\textsuperscript{2+} concentration.

The COOH-terminal, or tail, region of BBMIs and an equivalent region in invertebrate myosin I\(^{\text{S}}\)s have a high net positive charge and have been shown to bind with high affinity to negatively charged phospholipids (Adams and Pollard, 1989). This interaction has been proposed as a means by which myosin I\(^{\text{S}}\)s associate with membranes in vivo (Miyata et al., 1989; Hayden et al., 1990; Doberstein and Pollard, 1992; Zot et al., 1992). The predicted protein for MMI\(_{\alpha}\) also contains a COOH-terminal region with a high net positive charge and a predicted pI of 10.25. In comparison, the pI's of the equivalent regions in chicken and bovine BBMI are 10.41 and 10.44, respectively. The similarity within this region extends beyond a simple summation of charge. Alignment of this region with those of chicken and bovine myosin I reveals conserved amino acid stretches that may reflect shared functional domains (Fig. 1 B).

There are also major structural differences between MMI\(_{\alpha}\) and most of the myosin I\(^{\text{S}}\)s identified in Acanthamoeba and Dictyostelium. Unlike these myosins, yet similar to BBMIs and the Dictyostelium actin-based motor A (Titus et al., 1989), MMI\(_{\alpha}\) does not encode an ATP-independent actin binding site (Pollard et al., 1991).

### Expression Pattern of MMI\(_{\alpha}\), MMI\(_{\beta}\), and MMI\(_{\gamma}\) mRNAs

Northern blot analysis of the expression of MMI\(_{\alpha}\) mRNAs in various adult murine tissues reveals messages of 4.1, 4.9, and 6.5 kb that are most prominently detected in the brain, lung, and liver (Fig. 3). These messages are also expressed in the heart and testes, but are not detectable or are expressed at very low levels in the kidney, small intestine, skeletal muscle, adrenal gland, or spleen (data not shown). MMI\(_{\alpha}\) mRNA also shows variable expression in cultured cells and established cell lines. All three messages are present in the mouse neuroblastoma N115 line, the neural precursor line C17, and the murine teratocarcinoma cell line P19, yet are not detectable in the pheochromocytoma-derived PC12 cell line. MMI\(_{\alpha}\) transcripts are not detectable in the astrocytic cell lines U251 and G26-24, nor in isolated astrocytes from P6 rat cerebellum (Hatten, 1985) (data not shown). All three messages are present in normal rat kidney fibroblasts and NIH-3T3 cells, while in the CHO cell line only the 4.1-kb message is detected (data not shown).

The three MMI\(_{\alpha}\) messages noted above are detected by probes representing stretches of the coding region of the gene. To evaluate the correspondence of the detected messages with the isolated cDNAs, a subcloned fragment from the 3' untranslated region of clone XCNG74a that is not contained within clone XCNG82a (see above) was used to probe total RNA from various murine tissues. As illustrated in Fig. 3 A (lane 7), this probe detects the 4.9- and 6.5-kb messages but not the 4.1-kb message. This indicates that alternate sites of polyadenylation appear to account for the size difference between the two smaller messages. These findings also demonstrate that the 6.5-kb message contains the longer 3' untranslated segment, but the additional modifications necessary to generate this message are unknown.

MMI\(_{\beta}\) and MMI\(_{\gamma}\) display patterns of mRNA expression that are different both from each other and from MMI\(_{\alpha}\). In PC12 cells, a labeled pGMMI\(_{\beta}\) probe hybridizes to a single 5.0-kb message. This band is detected in all tissues and cell lines that express MMI\(_{\beta}\) mRNA. In tissue an additional 6.9-kb message is also detected with pGMMI\(_{\beta}\). The pGMMI\(_{\gamma}\) probe also detects a complex pattern of mRNAs, even at high stringency (0.2x SSC, 60°C). Hybridization to at least two messages of 5.2 and 70 kb is observed, although, as for MMI\(_{\beta}\), in PC12 cells only the 5.2-kb band is detected. The tissue and cell line distributions of MMI\(_{\beta}\) and MMI\(_{\gamma}\) mRNAs are distinct. MMI\(_{\beta}\) message is widely expressed: it is found in adult lung, kidney, heart, small intestine, adrenal gland, skeletal muscle, and P2 cerebral cortex (Fig. 4), as well as testes, spinal cord, and the adult cerebral cortex (data not shown). The 5.0-kb MMI\(_{\beta}\) message is also present in numerous cell lines of neural, glial, and fibroblastic origin (Fig. 4 B). MMI\(_{\gamma}\) mRNA, though not as widely expressed as MMI\(_{\beta}\), is found in lung, kidney, small intestine, and adrenal gland, and in the adult cerebral cortex and spinal cord (data not shown). MMI\(_{\gamma}\) is present at relatively low levels in the liver, heart, skeletal muscle, and P2 cerebral cortex. It is also not detected in several cell lines in which MMI\(_{\beta}\) is found: NIH3T3, U251-1, and P19 cells.

### Developmental Expression of MMI\(_{\alpha}\) mRNA in Neurons of the Postnatal Mouse Brain

Examination of MMI\(_{\alpha}\) mRNA in the postnatal mouse brain by Northern blot analysis points to a developmental regulation of the steady-state levels for these messages. As shown in Fig. 3 B, all three messages reach a peak at postnatal day 2 (P2) and decline thereafter to relatively low levels in the adult (Fig. 3, A and B). In the cerebellum, a peak of expres-
Figure 3. Tissue distribution and development regulation of MMI\textalpha mRNAs. 15 \mu g of total RNA from murine tissues or cell lines was analyzed by Northern blot hybridization with a 789-bp XhoI-EcoRI probe including amino acids 950-1079, except for the sample in lane 7. Unless indicated, tissue is from adult animals. 28S and 18S rRNAs are indicated by arrowheads. (A) Lane 1, P2 cerebral cortex; lane 2, adult cerebral cortex; lane 3, N115 neuroblastoma cell line; lane 4, lung; lane 5, testes; lane 6, liver; lane 7, lung. The RNA in lane 7 was hybridized with a probe containing only 3' untranslated nucleotides 3638-3998 that are contained within clone \lambda CNG74a and not clone \lambda CNG82a. (B) Total RNA from developing mouse cerebral cortex. Lane 1, P0; lane 2, P2; lane 3, P6; lane 4, P10; lane 5, adult.

Development occurs at P6, whereas the amount of message present in the adult is below detection by this approach (data not shown).

To provide further information regarding the cellular, regional, and developmental distribution of MMI\textalpha mRNA in the brain, we carried out in situ hybridization. Paraffin sections of brain tissue from mice ages P0 through adult were hybridized with a digoxigenin-labeled 400-nt cRNA probe containing both head and calmodulin domain sequence. Identical staining patterns are seen with an 800-nt probe against tail sequence. No staining is observed in the presence of excess unlabeled probe or with sense probes (data not shown).

In adult mice, MMI\textalpha mRNA is widely distributed in neurons throughout the brain. Regions examined included the cerebral cortex, hippocampus, and dentate gyrus, subcortical areas, brainstem, and cerebellum. All stained cells contain large nuclei and a thin ring of cytoplasm (Fig. 5b), characteristic of neurons; an extensive search did not reveal reaction product in any cells of glial or astrocytic morphology. However, reaction product is found in cells of the choroid plexus. Comparison with adjacent sections stained

Figure 4. Tissue and cell line distribution of MMI\beta and MMI\gamma. 10 \mu g of total RNA from rat tissue or cell lines was analyzed by Northern blot hybridization with probes from the indicated 1.2-kb PCR product. The positions of 28S and 18S rRNA are denoted by arrowheads. (A) Lane 1, lung; lane 2, liver; lane 3, kidney; lane 4, heart; lane 5, small intestine; lane 6, adrenal gland; lane 7, hind leg skeletal muscle; lane 8, P2 cerebral cortex; lane 9, PC12 cells. (B) Lane 1, PC12 cells; lane 2, NIH 3T3 cells; lane 3, U252-1 astrocytoma; lane 4, C17 cells; lane 5, P19 cells; lane 6, P19 cells + 5 \mu M retinoic acid.
Figure 5. Photomicrographs of in situ localization of MMIA mRNA in adult mouse brain. These coronal sections from an adult mouse depict the in situ localization of MMIA mRNA in the cerebral cortex (a), high magnification micrograph of the cerebral cortex (b), hippocampus (c), subcortical diencephalon (d), cerebellum (e), and brainstem (f). The color reaction in the section of the cerebellum was allowed to develop longer in order to enhance the labeling intensity of the granular layer. MH, medial habenula; LH, lateral habenula; Th, thalamus; M, molecular layer; p, Purkinje cell layer; G, granular layer; AP, area postrema; NTS, nucleus of the solitary tract; 12N, hypoglossal nucleus; Gr, nucleus gracilis; Cu, nucleus cuneatus.

with hematoxylin and eosin (data not shown) reveals that nearly all neurons appeared labeled, although the intensity of staining showed regional differences. For instance, in the cerebral cortex, the intensity of reaction product is greater in neurons of layer three than in neurons of the other cortical layers (Fig. 5 a). Also, in the cerebellum Purkinje cells and neurons of the deep nuclei stain much more intensely for MMIA mRNA than do granule neurons. In contrast, labeling in the hippocampus and dentate gyrus appears to be uniformly strong in all neuron layers.
Examination of P0, P2, P7, and P12 brains reveals that the proportion of positively stained neurons does not vary significantly during central nervous system maturation. However, there are both regional and temporal changes in expression of MMIα mRNA during development. In the hippocampus a uniformly intense level of staining is detected from P0 through adult. In areas including the cerebral cortex and cerebellum, however, the overall level of staining declines with development. For example, in consonance with Northern blot data, neurons within the cerebral cortex generally show peak levels of expression between days P2 and P7. In cerebellum at day P2, granular neurons exhibit intense and uniform labeling, whereas neurons in the Purkinje layer do not stain uniformly. This situation progressively changes with development so that, by P12 and onward, staining in Purkinje cells is uniformly intense and a relatively light staining pattern is observed in all cerebellar granular neurons (Fig. 6).

Hybridization analysis also indicates expression of MMIα mRNA in at least certain neuronal precursors. In the developing mouse cerebellum, neuroblasts migrate to form the external granular (or germinal) layer (EGL) where they continue to divide postnatally and give rise to granule, stellate, and basket cells (Jacobson, 1991). Throughout the developmental period examined (P0 to P12) all cells in the EGL label with probes to MMIα. However, staining was more intense in cells of the superficial layers of the EGL (Fig. 6). It is in this region of the EGL where granule cells continue to undergo mitosis and are just beginning to extend their bipolar processes.

**Discussion**

**Evidence for a Family of Mammalian Myosin I Genes**

The characterization of MMIα, MMIβ, and MMIγ provides strong evidence for the existence of three novel mammalian myosin I genes. These are the first vertebrate myosin I genes described that are expressed outside the intestinal brush border. The classification of these predicted proteins as myosin Is is based on both a high degree of overall homology and the presence of highly conserved domains: some of these are shared by all myosins and others are unique to myosin Is. The latter point is exemplified by the conserved residues, QA.yaRALAK.iYISR, first described as adjacent to the Acanthamoeba myosin I phosphorylation site (Pollard et al., 1991). Given the presence of multiple myosin I genes in at least two invertebrates, the existence of a family of mammalian myosin I genes is not unexpected.

We screened 42 subcloned PCR products to identify these three novel myosin I genes. While this is not an exhaustive search for myosin I family members, this result may suggest that these are the only products that can be obtained from these cell sources given the constraints of the PCR strategy we used. These constraints also would prevent the amplification of unconventional myosins like NinaC (Montell and Rubin, 1988) and the high molecular weight myosin I from Acanthamoeba (Horowitz and Hammer, 1990).

MMIβ may be the rat homologue of a newly identified myosin I purified from bovine adrenal glands (Barylko et al., 1992). 12 residues, LSVDFTEDVE, have at least 75% identity with a microsequenced fragment from the bovine myosin. A more recent paper from this group (Wagner et al., 1992) provides localization data for this protein in many tissues and cell types that overlap with expression of the MMIβ transcript.

**Expression Pattern of MMIα, MMIβ, and MMIγ**

MMIα is highly expressed in the brain, lung, and liver, while
MMIα and MMIβ are detected in diverse tissues including the lung, kidney, small intestine, adrenal gland, and brain. These patterns of expression are distinct from one another and also substantially different from that of BBMI, which appears by both Northern blot and immunohistochemical analysis to be expressed only in the brush border of the small intestine (Hoshimaru et al., 1989; Bikle et al., 1991). The very different cellular distribution of these messages is consistent with the possibility that each of these products performs separate and unique functions.

The various messages detected by probes derived from MMIα, pGMMIβ, and pGMMIγ point to a further complexity within the mammalian myosin I family. One potential concern in interpreting these results is the crossreactivity that may occur by using myosin head sequence as the cDNA probe. However, hybridization was carried out at high sensitivity, and thus we expect that the bands correspond to alternate transcripts of the same gene or to ones that are highly homologous. This is supported by the different tissue distribution detected with probes to the three different genes. For MMIβ and MMIγ, this is further supported by the S1 nuclease protection results. For MMIα, the chance of crossreactivity is most unlikely because similar results were obtained using multiple probes, including those containing tail and 3′ untranslated sequences.

Three messages are detected with MMIα probes. Two of the messages appear to differ by alternate polyadenylation sites. The modifications responsible for generating the largest 6.5-kb message may include an additional coding sequence representing a separate functional domain, or this band may represent unprocessed nuclear RNA. An example of a myosin gene encoding multiple proteins is ninaC, in which the two transcripts differ by the presence of the COOH-terminal membrane binding domain (Porter et al., 1992). Using probes derived from pGMMIβ and pGMMIγ, we have detected a complex pattern of bands primarily in tissue; the cell lines examined typically displayed only a subset of these messages, suggesting that certain transcripts may be differentially expressed within multiple cell types in a single tissue.

**MMIα Encodes a Protein Structurally Similar to BBMI**

The cDNAs we isolated for MMIα encode a myosin I containing three functional domains: a canonical myosin head that contains sites for ATP hydrolysis and actin binding, a neck that possesses sites for calmodulin binding, and a positively charged tail that is likely to participate in membrane binding.

Calmodulin is the light chain for BBMI, for the unconventional myosins p190 (Espreafico et al., 1992), and probably for two other vertebrate myosin I proteins that have recently been purified (Coluccio, 1991; Barylko et al., 1992). Association of calmodulin with the heavy chain has been shown to regulate the activity of BBMI (Coluccio and Bretscher, 1987; Collins et al., 1990; Mooseker et al., 1989; Swanljung-Collins and Collins, 1991). The unconventional myosins p190 (Espreafico et al., 1992), dilute (Merce et al., 1991), and yeast myo2 (Johnston et al., 1991), as well as BBMI, all encode a region in the neck of the protein that contains four to six related sequences of ~23 amino acids. These elements are homologous to the well-characterized calmodulin binding domain of neuromodulin (Chapman et al., 1991a,b). Like neuromodulin, BBMI and p190 bind calmodulin in the absence of Ca2+. Within the analogous neck region, the predicted MMIα protein contains five calmodulin binding sites, and thus also probably binds calmodulin in a Ca2+-independent manner. While these five sites possess the common motifs that appear necessary for calmodulin binding, each is more closely related to the homologous site in BBMI than to any of the other four sites within MMIα (and vice versa). This suggests that each site has been uniquely conserved. Moreover, these differences between sites may be reflected by biochemical data for BBMI that demonstrate a complex interaction between Ca2+ concentration, the number of calmodulins bound, and motor activity.

The neck region of MMIα also encompasses a 29 amino acid stretch that has high homology, based on sequence and location within the molecule, to a 29 amino acid calmodulin binding insert encoded by a rare transcript of chick BBMI (Halsall and Hammer, 1990). Interestingly, this stretch is encoded by all three MMy α cDNAs we have isolated and sequenced. However, it is possible that there may be additional splice products that vary in this region.

The COOH-terminal region of the predicted MMIα protein contains a high net positive charge. In both BBMI and Acanthamoeba myosin I's this region is also highly basic and has been implicated in the binding of these proteins to the plasma membrane. This interaction is mediated at least in part by a high affinity for negatively charged phospholipids found on the inner leaflet of the membrane (Adams and Pollard, 1989; Zot et al., 1992). Thus, MMIα protein would be expected to link the actin cytoskeleton to the cell membrane. Comparison of MMIα to both bovine and chicken BBMI reveals conserved domains within the tail (Fig. 1 B). Interestingly, 65% of these residues are not charged. In contrast, overall charge, but not specifically these domains, is conserved between MMIα and the equivalent regions in Acanthamoeba or Dictyostelium myosin I's.

We have considered here as myosin I all myosins that possess the positively charged membrane binding domain and do not contain any heptad repeats for dimerization, including all the Acanthamoeba and Dictyostelium myosin I's and BBMIs. This excludes such molecules as dilute, ninaC, and myo2, which have been classified within a broader category as "unconventional" myosins (Cheney and Mooseker, 1992). Even this narrow classification for myosin I may permit potential subdivisions. The 65% overall homology of MMIα to BBMI, as compared with 71% between the chick and bovine BBMIs, suggests a high degree of relatedness. This contrasts with the lower degree of homology (47-49%) that pGMMIβ and pGMMIγ show to the head domain of BBMI and to each other. Thus, MMIα and the BBMIs can be viewed as a subfamily within the myosin I family as outlined (Espreafico et al., 1992), while MMIγ and MMIα would presently each be single members of separate groupings. Whether these similarities and differences extend to function remains to be determined.

**Expression of MMIα in the Nervous System**

In situ hybridization and Northern blot analysis reveals that MMIα mRNA is expressed in the mouse brain, from the neonate to the adult. Whereas virtually all brain neurons ex-
press MM10 mRNA, no message is observed in glial cells or in glial cell lines. We cannot, however, rule out the presence of MM10 mRNA in microglia or other relatively sparse populations of neuronal cells within the brain.

Although the MM10 message is present at all postnatal stages, expression varies both developmentally and regionally. For example, in situ hybridization in cerebellar granular neurons indicates that MM10 mRNA declines substantially from a peak between P7 and P12 to lower levels in the adult, whereas staining in hippocampal neurons remains equally strong throughout development. Northern blots reveal a general postnatal developmental decline in the expression of MM10 mRNA in the brain. Because most gliogenesis (in contrast to neurogenesis) occurs in the neonate, we suggest that the Northern blot data reflect summation of the actual decrease in average message levels per neuron and the dilution concomitant with the increasing relative percentage of glial RNA.

In addition to postmitotic neurons, neuroblasts in the external granular layer of the cerebellar cortex also stain for MM10 mRNA. These cells continue to divide postnatally; those fated to become granular neurons (the great majority) migrate past the Purkinje cells to form the internal granular layer (Altman, 1972). Interestingly, MM10 mRNA expression is highest in the outermost layer of the EGL (in contrast to the inner layers), which contains those cells that are still dividing and just beginning to extend bipolar processes. We do not yet know whether MM10 is expressed in other neuroblasts and how early this expression commences. It will be of considerable interest to explore these questions through examination of the embryonic brain.

We began our search for novel myosin I genes with the hypothesis that one or more of these motor proteins may participate in processes critical to neuronal development and function such as cell migration, neurite outgrowth, and vesicular transport. The presence of MM10 in all neurons examined and their absence from glial cell are consistent with these roles. The high sequence homology of MM10 with BBM1, as well as the high level of MM10 mRNA in neuroblasts just beginning to extend processes, may suggest a role for MM10 at the leading edge of these processes creating motive force between the plasma membrane and the actin cytoskeleton. Whether MM10 can function as a motor for growth cone–like motility is the subject of ongoing investigation.

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