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A UNIQUE CELLULAR MYOSIN II EXHIBITING DIFFERENTIAL EXPRESSION IN THE CEREBRAL CORTEX

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Clones possessing inserts of brain myosin II have been obtained by screening a rat brain cDNA expression library with a polyclonal antibody, raised against myosin II from the mouse neuroblastoma cell line, Neuro-2A. A partial sequence comprising the 3' coding and non-coding regions of the myosin message has been determined which is markedly different from other myosin sequences. The derived amino-acid sequence comprises the C-terminal 90 amino acids: VSS(PO_4)LKNKLRRGDLPFVVTRRLVRKGTLELS(PO_4)DDDDESKASLINETQPPQCLDQQLDQQ LDQLFNWPVNAGCVCGWGVEQTQGEEAVHKCRT(CO_2H). This sequence encompasses regions homologous to both the casein kinase II and protein kinase C heavy-chain phosphorylation sites. The non-helical "tail-piece" is considerably longer (an additional 39 amino acid residues) than found in other myosins. Northern blot analysis demonstrates this myosin II message to be unique to cerebral cortex, with no expression in all other non-cortical brain regions and peripheral tissues tested. Our results suggest functional diversity for myosin II isozymes within the brain.

Two-headed myosin (myosin II) is found in almost all eukaryotic cells where it exhibits roles in cell motility and cell division. Brain myosin II has been isolated (1-3) and its biochemical properties have been studied in some detail (3-7), but the diversity and distribution of isozymic forms of brain myosin remain unknown. While roles in growth cone migration and neurotransmission have been proposed (1,2,8,9) for brain myosins, these ideas remain largely untested. As a first step in an approach designed to answer some of these questions, we have raised and affinity-purified a polyclonal antibody against myosin II purified from a neuronally-derived cell line (20). We have used this antibody to screen a rat brain cDNA expression library with the ultimate objective of sequencing brain myosin and using partial sequence information to create antisense probes with which to assess neuronal myosin function. When one performs such a screening on clones possessing short inserts, the clonal selection process is essentially monoclonal, even when using a polyclonal antibody. Here, we report that by using this procedure, we have been able to clone and sequence the 3' coding and non-coding region of cDNA from an unusual myosin II isozyme, which appears to be differentially expressed within the cerebral cortex.

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Materials and Methods

A rat brain cDNA library in λZap-II (Stratagene) was screened using an affinity-purified polyclonal antibody raised against myosin extracted from the mouse neuroblastoma cell line, Neuro-2A. A total of 16 positive clones were selected, which remained positive following two rescreenings, from a total of $5x10^5$ plaques. The production, affinity purification and characterization of the antibody used has been described elsewhere (Miller, Bower, Kensler, Levitt & Chantler; Manuscript submitted for publication). Briefly, a polyclonal antibody was raised by injection of purified neuroblastoma myosin into rabbits and the resulting antiserum was affinity-purified by sequential passage over striated muscle myosin (flow-through retained) and brain myosin (bound fraction retained) affinity columns, prior to specific elution of antibody bound to neuroblastoma myosin heavy-chain after incubation of the purified antiserum with neuroblastoma myosin blotted onto nitrocellulose. The resulting antibody recognized brain and neuroblastoma myosins with high affinity.

Inserts in pBluescript were sequenced by double stranded methods using the dideoxy chain termination method (16) using Sequenase 2.0 (U.S.B.). Sequence analysis was executed using the Genepro program, Version 4.20 (Riverside Scientific Enterprises, CA.) employing the GeneBank database, Version 62, for sequence comparisons.

Probes were randomly labelled with α-[^{32}P]dCTP (specific activity of 3000 Ci/mmole, ICN Biomedicals, Irvine, CA) by standard techniques (15) using the Klenow fragment of DNA polymerase I ("Prime-a-Gene" labelling system, Promega). RNA was prepared from rat brains by acid guanidinium thiocyanate-phenol-chloroform extraction (17). RNA was size-fractionated by formaldehyde gel electrophoresis and transferred to a nitrocellulose membrane (Micron Separations Inc.) during Northern blot analysis according to standard procedures (15). Prehybridization was in 5x Denharts, 50% deionized formamide, 50mM Tris, pH 7.5, 0.8M NaCl, 10% dextran sulfate, 100 μg/mL salmon sperm DNA at 42^O for 2 hours. Hybridization took place at 42^O overnight with labelled probe in the same buffer. The stringency of the final wash was 0.1x SSC, 0.1% SDS at 65^O for 20 minutes. The filter was exposed for 48 hours to X-ray film (Kodak X-AR2) at -70^O with intensifying screen.

Results and Discussion

We obtained 16 positive clones upon screening a rat brain library (Stratagene) with our anti-neuroblastoma myosin antibody. C4 and CT1 are the names given to two of these clones which possessed a substantial portion of the 3' non-coding region. C4 and CT1 are 0.26 and 0.54 kB in size respectively, and both extend into the coding region for the carboxyl terminus of the heavy-chain as well as overlapping within portions of the 3' non-coding region (see Figure 1 for schematic representation). C4 is contained entirely within the CT1 sequence.

The combined sequence obtained from both C4 and CT1 is shown in Figure 2. The assignment of these sequences to the 3' end of myosin cDNA (Figure 1) is confirmed through the substantial sequence homology (94%) which exists between the 3' end of CT1 and the 3' non-coding region of chicken cellular myosin cDNA (10), 111 nucleotides being identical in both sequences (Figure 2). This assignment was further substantiated through knowledge of the 22 amino acid residues surrounding a serine residue known to be phosphorylated by casein kinase II (12). This sequence, fully contained within our own, was originally determined by protein chemistry methods using bovine brain myosin II (12), and enabled us to establish the reading frame of clone CT1 and so determine the additional 39 residues linking this known sequence to the carboxyl tip of the tail (Figure 2). At the 5' end of our sequence, there is a region which is completely homologous at the amino acid level with the peptide sequence shown to be phosphorylated by protein kinase C in platelet myosin (21).

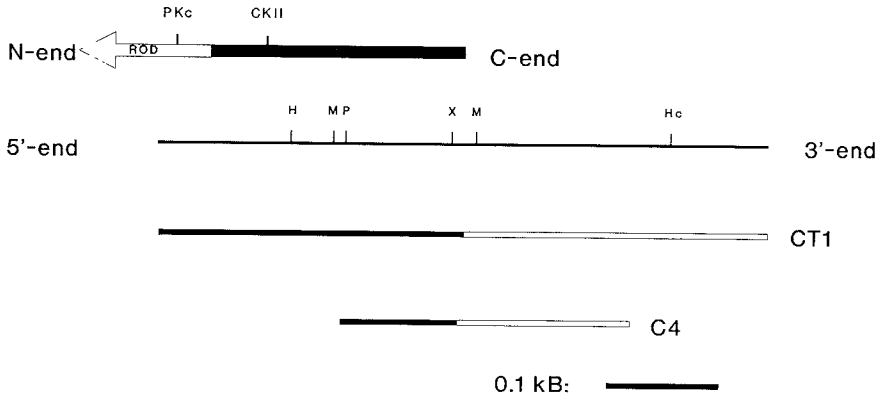

Fig. 1. Schematic representation and partial restriction map of rat brain non-muscle myosin II cDNA clones, CT1 (537 bp) and C4 (260 bp). The C-terminal region of the myosin heavy-chain protein is shown above for orientation purposes; the non-helical tail region is indicated by solid shading and the putative heavy-chain phosphorylation sites indicated (casein kinase II site within the tail-piece; protein kinase C site just within the rod). Below the protein map is a partial restriction map for the cDNA clones: Sites marked are H, *Hind* III; M, *Mae*I; P, *Pvu*II; X, *Xho*II and Hc, *Hinc*III. Below are the cDNA clones, CT1 and C4. Solid shading within the cDNA clones represent coding regions for parts of the non-helical tail; absence of shading represents the 3' untranslated regions. The CT1 sequence extends to the poly-A tail.

```
          PKc                                                  LPR
GTC AGC TCC CTA AAG AAC AAG CTC AGG CGC GGG GAC CTG CCC
Val Ser Ser Leu Lys Asn Lys Leu Arg Arg Gly Asp Leu Pro

TTT GTG GTG ACC CGG CGG CTG GTG CGG AAG GGC ACC CTG GAG
Phe Val Val Thr Arg Arg Leu Val Arg Lys Gly Thr Leu Glu

    CKII                                          #
CTG TCG GAT GAT GAT GAT GAA TCC AAA GCT TCC CTA ATC AAT
Leu Ser Asp Asp Asp Asp Glu Ser Lys Ala Ser Leu Ile Asn

GAA ACC CAG CCC CCC CAA TGC CTA GAT CAG CAG CTG GAC CAG
Glu Thr Gln Pro Pro Gln Cys Leu Asp Gln Gln Leu Asp Gln

CTC TTT CAT TGG CCA GTG AAT GCA GGG TGT GTG TGT GGG TGG
Leu Phe His Trp Pro Val Asn Ala Gly Cys Val Cys Gly Trp

GGG GTG GAG CAG ACA CAA GGG GAG GAG GCA GTG CAC AAG TGC
Gly Val Glu Gln Thr Gln Gly Glu Glu Ala Val His Lys Cys

AGG ATC TAA AACGGAGGCAAGGGCTAGGGATACAGCTCAGTGGTAGAGCACT
Arg Ile  *

GGGAGGCCCTGGGTTCTATCCCCAAGTGTTTTTCTTATCACCGTGGTCATATGTC

AAGGAGGAGGAACTTAAGGAGGAGGCGCTTACATTGGCTCCCAGTTTGATCAGTC

    H
ATGTACCAGGGTTGGATTCGTTATTGTGCGCCGTTGACTTAGAGATATTAAAAAC

CCTTAAATCTGCACAGATATATGTAAGGGCCTTGTATCTCTCGTAATAAGTAATT

AAGAAAAAAAAAA 3'-end
```

Fig. 2. Nucleotide and predicted amino acid sequence for the rat brain non-muscle myosin II cDNA clones, CT1 and C4. The complete nucleotide sequence shown is that of CT1. The C4 clone is indicated within this by the continuous line below the nucleotide sequence. Amino acid residues are indicated underneath the appropriate nucleotide codons. The stop codon, immediately following the C-terminal isoleucine, is marked with an asterisk. The *amino acid* sequence previously determined by protein sequencing methods (12), is indicated by bold lettering. Murakami et al. (12) were unable to assign one amino acid in their sequence; the unknown amino acid in our isoform is a leucine residue and is marked by a sharp sign. The serine residue phosphorylated by casein kinase II (CKII) is found within this region. The serine residue phosphorylated by protein kinase C in platelet myosin (21) and the adjacent pair of amino acids in either direction, are also conserved in the brain myosin sequence. The putative serine phosphorylated by protein kinase C is marked (PKc). By comparison with other myosin sequences, the last proline residue which terminates the rod region of the myosin tail is also located (LPR). *Nucleotide* residues 3' to the residue marked with an **H** are 94% homologous with those of the chicken cellular myosin non-coding cDNA sequence (10).

Despite such similarities, a large stretch of cDNA sequence appears to be unique to brain myosin. The region encoded by C4 is very different from the 3' non-coding regions of both chicken cellular (10) and human fibroblast and macrophage (11) myosins. Figure 3A compares the tail-piece amino-acid sequences from chicken cellular (10) and human macrophage (11) myosins to our brain neuronal myosin. Whereas these other cellular myosins show strong similarities to each other, the brain myosin sequence is strikingly different. In addition to a significantly lower degree of amino-acid homology in the region of overlap, the brain myosin sequence possesses an extended tail-piece (Figure 3A) containing 39 additional amino-acids not found in the other myosins. A hydropathy plot of the tail-piece (Figure 3B) shows the presence of a hydrophobic section flanked by stretches of hydrophilic sequence. We confirm that the hydrophobic repeat typical of α-helical coiled-coils is absent, suggesting that the entirety of this "tail-piece" (10) is non-helical, a feature found in other non-muscle myosins but not in myosins isolated from striated muscle.

Evidence for the differential expression of two different non-muscle myosin heavy-chains in various tissues has been obtained by probing Northern blots with two distinct cDNA clones from chicken fibroblasts (13). These authors demonstrated that one form of myosin is substantially enriched in brain as opposed to other tissues, whereas the other form is predominant in spleen. The presence of additional myosin isozymes could not be ruled out, however. In order to determine whether the sequence we had obtained was related to either the "brain-type" or "spleen-type" forms of myosin II, noted by Masaki and colleagues (13), or represented a third distinct type of non-muscle myosin, possibly with a restricted distribution, we performed Northern blots on total RNA from different tissues using [^{32}P]-labelled C4.

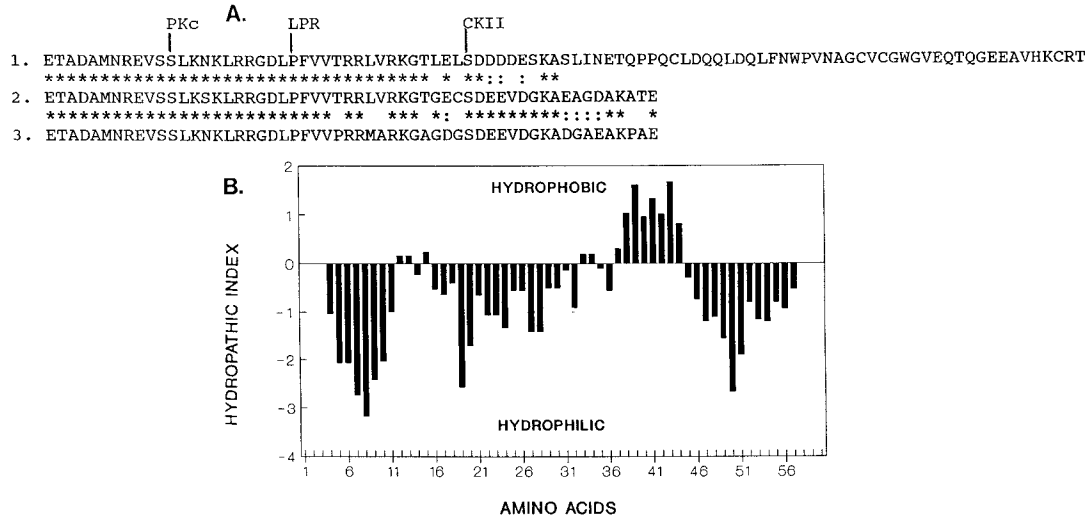

Fig. 3. A. Comparison of the carboxy-terminal "tail-pieces" for three cellular myosins. I: brain cortical myosin (this paper). II: chicken intestinal cellular myosin (10). III: human macrophage myosin (11). Single letter amino acid code used throughout. Asterisks between residues of adjacent sequences indicates amino-acid identity. Double dots between residues of adjacent sequences indicates conserved homology.

B. Hydropathy plot of the C-terminal tail-piece from brain cortical myosin (I, above). The relative hydrophobicity of this 60 amino-acid sequence is calculated according to the procedures of Kyte and Doolittle (19), using a seven amino-acid window.

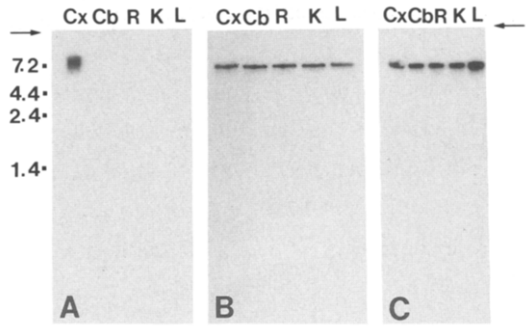

Fig. 4. Localization of rat brain myosin II sequence encoded by the C4 probe, to brain cortex. Shown here are Northern blots, visualized through the use of randomly labelled cDNA probes (α-[^{32}P]dCTP) for C4 (A), and the 5' (B) and 3' (C) regions of chicken brush border myosin (chicken cellular myosin cDNA probes (10) kindly provided by Drs. Bob Adelstein & Mary-Anne Conti, Molecular Cardiology, N.I.H., Bethesda, MD.). Arrows indicate the location of sample loading. Numbers on LHS (in kilobase units) represent the location of non-radioactive size markers.

Lanes contain 15 μg of total RNA from rat liver (L), kidney (K), brain cortex (C), cerebellum (Cb) and a preparation of brain from which cortex had been selectively removed (R). Non-radiolabelled RNA standards were used to define the range shown on LHS. RNA preparation and Northern blot procedures are described in Materials and Methods.

Note that whereas the 5' and 3' chicken cellular myosin probes hybridize to a single myosin mRNA (7.2 kB) from all sources tested, the C4 probe only hybridizes with a single 7.2 kB component present in brain cortex and does not hybridize with RNA from cerebellum or a preparation of brain from which cortex had been dissected away.

Using total RNA extracted from rat brain, C4 hybridized with a single band ~7.2 kB in size, a pattern consistent with the expected size range for a cellular myosin mRNA (6.9 - 7.3 kB). No hybridization was observed with RNA from kidney or liver (Figure 4), a result in contrast to that observed by Masaki and colleagues (13) who found, by Northern blot analysis, evidence for their "brain-type" myosin in these organs. Most surprisingly, when RNA was isolated from different brain regions (cortex, cerebellum or a preparation of brain from which the cortex had been dissected away), C4 only hybridized with cortex at the stringency employed (Figure 4). By contrast, chicken non-muscle myosin cDNA probes (kindly provided by Drs. Bob Adelstein and Mary-Anne Conti, Molecular Cardiology, N.I.H., Bethesda, MD.) hybridized with all of our RNA preparations from all sources tested, under the same conditions of stringency (Figure 4).

The evidence presented here suggests the presence of a hitherto unknown isoform of non-muscle myosin II, unique to the brain cortex. Because of our antibody affinity purification protocol, this isoform is probably of neuronal origin. The unusual extended tail-piece may be of functional significance. It could be globular in form, with the hydrophilic flanking sequences enveloping the hydrophobic core. Alternatively, the clear demarcations of these polar and non-polar regions seen in the hydropathy plot make it conceivable that, whilst the analysis does not lend support to a transbilayer structure (18), this region may interact with the plasma membrane in some way. A region for membrane association within brain myosin II would be consistent with the known requirement for the presence of a non-

ionic detergent during the early stages of brain myosin II preparation, as well as the ultrastructural localization of neuronal myosins immediately adjacent to the dendritic plasma membrane (Miller, Bower, Kensler, Levitt & Chantler. Manuscript submitted for publication). The 3' non-coding region of the brain cortical myosin described here differs from equivalent non-coding sequences described for other cellular myosins (10,11), a feature suggestive of specific functional control for such regions are thought to be involved in the regulation of expression (14). The substantial homology exhibited by this brain myosin cDNA sequence for chicken cellular myosin, both within coding and non-coding regions which flank unique sequence, suggests that the expression of this isoform may involve alternative splicing of a conserved, multifunctional gene for myosin II. Exactly why neurons of the brain cortex require expression of a specific myosin isoform is open to conjecture. It is plausible that myosin may actively participate in key elements of neuronal functioning, such as neuronal plasticity, a role which may necessitate regional specialization.

Acknowledgments

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