FEBS Letters 348 (1994) 244–248

FEBS 14227

Functional analysis of individual brain myosin II isoforms through hybrid formation

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Received 4 May 1994; revised version received 3 June 1994

Abstract

We have used a scallop hybrid myosin test system in an attempt to determine the regulatory properties of an individual myosin II isoform from rat brain. The complete coding region of cDNA corresponding to a regulatory light chain isoform previously shown to be expressed in brain [Feinstein, Durand and Milner (1991) Mol. Brain Res. 10, 97–105] was ligated within the prokaryotic expression vector, *p*AED4, overexpressed in bacteria, and the purified light chain incorporated within a scallop hybrid myosin. Actin activation was calcium insensitive for all hybrids tested, irrespective of whether light chain phosphorylation had taken place before, or subsequent to, hybrid formation. We discuss the implications of these results, including the possibility that these results constitute evidence for a myosin II isoform within brain that is regulated at the level of the thin filament. In addition, evidence is presented for the presence of an additional, novel isoform of regulatory light chain expressed in rat brain.

Key words: Brain myosin; Myosin light chain; Hybrid formation; Light chain cDNA sequence

1. Introduction

The interaction between myosin II and actin may be controlled by actin-linked or myosin-linked mechanisms of regulation [1]. In the latter case, light chains present within the heads of regulatory myosins are able to control the level of interaction between actin and myosin [2]. This is in contrast to thin filament linked regulation where the major regulatory components are associated with the actin containing thin filament [3]. Reversible removal of regulatory light chains (RLCs) from scallop myosin [4,5] has facilitated the formation of hybrid myosins, permitting classification of the regulatory system from which the guest RLC was obtained [6–8] and creation of an in vitro test system for mutant light chains [9–11]. Whereas RLCs from striated muscle myosins could not restore calcium sensitivity to desensitized scallop myosin [6–8], RLCs from myosins controlled by direct calcium binding or by RLC phosporylation could restore regulation [7,8,12].

When myosin II is isolated in bulk from brain, the preparation is necessarily representative of a mixture of isoforms, variously present in neurons, glia and vascular smooth muscle cells [13–16], thereby making direct functional assessment of individual myosin isoforms impossible. An alternative approach in achieving this goal is possible through the construction and testing of hybrid myosins, using individual RLC isoforms which are produced by overexpression in bacteria from single clones. To this end we have screened a rat brain cDNA library so as to clone and express RLC isoforms previously located in brain [17]. The purified RLC was tested for regulatory capability as a component of scallop hybrid myosin, both as a function of calcium and of RLC phosphorylation. Direct sequencing evidence for the presence of at least three RLC isoforms from rat brain was obtained, including evidence for a novel isoform. A preliminary account of this work has been presented earlier [18].

2. Materials and methods

Clones (5×10^5) from a rat brain cDNA expression library (Stratagene) were screened using two 3′ end-labeled (End-All Labeling Biosystems, IBI) 36-mer probes designed to hybridize to conserved sequences found in both of two RLC isoforms located in brain [17,22]. The oligonucleotides were 5′-CAGGAGTTCAAAGAGGCCTTCAACATGATCGACCAG-3′ and 5′-GAGTTCACGCGCATCCTCAAGCACGGAGCGAAAGAC-3′, corresponding to amino acids 32–43 and 158–169, respectively, in both RLCs. Final washes were performed in 0.5 × SSC and 0.2% SDS at 42°C. Inserts in *p*Bluescript (Stratagene) were sequenced using Sequenase 2.0 (U.S.B.) and the dideoxy chain termination method [19].

A RLC expression plasmid was created by cloning full length RLC cDNA into the expression plasmid *p*AED4 (courtesy of Dr. Don Doering, Whitehead Institute, Cambridge, MA). The full-length insert was obtained by DNA amplification (10 ng template DNA, 500 ng of each primer, 25 cycles of 95°C (1 min), 50°C (2 min) and 72°C (3 min) on a thermal cycler using 5′ (5′-AACCGCCCATATGTCGAGCAAAAAAGCA-3′) and 3′ (5′-TCAACAGGAATGTCAGCTGCAGCTCTTC-3′) primers. *Nde*I and *Pvu*II sites (underlined) are incorporated within the 5′ and 3′ primers, repectively. Both the amplification product and the vector were digested with *Nde*I/*Pvu*II and products isolated by agarose gel electrophoresis, then ligated together with T4-DNA ligase. It may be noted that creation of the *Nde*I site by in vitro mutagenesis facilitated ligation of the amplification product immediately in front of

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Abbreviations: EGTA, ethylene glycol bis(aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulphate; IPTG, isopropyl-*β*-thio-galactopyranoside; ELC, essential light chain; RLC, regulatory light chain; MLCK, myosin light chain kinase.

SSDI 0014-5793(94)00613-Z

the ATG start site and downstream of the T7 promoter. The completed expression plasmid was transformed into *E. coli* BL21 and the RLC insert resequenced so as to confirm the absence of any spontaneous mutation during amplification.

Overnight cultures of *E. coli* BL21 transformed with expression plasmid *p*AED4 containing the full length RLC cDNA were diluted 100-fold, then regrown to an OD_{600} of 0.5–0.6 before induction with IPTG (final concentration 0.4 mM). After 5 h, bacteria were pelleted and solubilized overnight in 8 M guanidine hydrochloride prior to ethanol precipitation and RLC purification as described previously [20].

Standard procedures were used to prepare intact and desensitized scallop myofibrils and for resensitization [5]. RLC phosophorylation was performed, either on the isolated light chain or on the hybrid myosin, by addition of myosin light chain kinase (MLCK) (courtesy of Dr. Deqin Li) in the following buffer: 150 mM KCl, 5 mM $MgCl_2$, 10 μM Tris pH 7.5, 1 mM DTT, 0.2 mM $CaCl_2$, 2.5 mM [γ-^{32}P]ATP, 10 μg/ml calmodulin, 10 mg/ml MLCK. Actin-activated Mg-ATPase rates were measured for thirty minutes, in the presence (0.2 mM) and absence of calcium, under standard conditions at low ionic strength (40 mM NaCl, 2 mM $MgCl_2$, 10 mM phosphate, 1.0 mM ATP, 0.1 mM EGTA, 3 mM NaN_3, pH 7.0) using ^{32}P release [21]. At the end of each assay aliquots were removed, centrifuged, washed and analyzed by SDS/acrylamide gel electrophoresis so as to ensure that no light chain dissociation had occurred during ATP hydrolysis. Densitometry of Fast green stained SDS/acrylamide gels was performed on all brain RLC hybrids by standard procedures [5].

The three sequences described here can be accessed from the EMBL data library as Z31721 (FY53), Z32518 (YU63) and Z32519 (TY21).

3. Results

Analysis of ten positive clones revealed direct sequencing evidence for the presence of three distinct RLC sequences in rat brain (Fig. 1). As anticipated, we found clones representing the sequences obtained by Feinstein and colleagues [17](our sequence FY53) and by Taubman and colleagues [22] (our sequence TY21). We were able to verify that the deduced amino acid sequence presented formerly [17] had lacked only a single N-terminal methionine and we were able to extend the cDNA sequence some 44 residues into the 5′ non-coding region. Regarding the sequence by Taubman and colleagues [22], TY21 contains three nucleotide insertions and two substitutions with respect to the revised version of the sequence from these authors (Genbank Accession no. X05566) and these are described in the legend to Fig. 1. In addition, we have obtained evidence for the existence of a third RLC isoform in brain (our sequence YU63, Fig. 1). This partial sequence enabled us to define differences from the two previously published sequences which are to be found, at both the nucleotide and amino acid levels, in the region of amino acids 63–68 (numbering as in ref. 22).

The complete cDNA sequence for RLC FY53 was ligated into the pAED4 expression vector (see section 2), overexpressed in *E. coli* BL21 and the purified light chain was hybridized to desensitized scallop myofibrils (Fig. 2). The brain RLCs could be phosphorylated either before (Fig. 2B,C; lanes 1 and 2) or after (Fig 2B,C; lanes 3 and 4) rebinding to form scallop hybrid myosin. The various products seen in Fig. 2 were assessed for calcium-dependent actin-activated MgATPase activity and results are presented in Table 1. The brain RLC isoform expressed from FY53 was not capable of restoring calcium sensitivity to desensitized scallop myofibrils, irrespective of the presence or absence of light chain phosphorylation and irrespective of whether phosphorylation took place on the isolated light chain or subsequent to myosin hybrid formation.

4. Discussion

Owing to the fact that a number of myosin II isoforms are found in brain [13–16], biochemical analysis on preparations of myosin II from brain necessarily measures properties representative of the whole population. Such characterization has demonstrated that the actin-activated MgATPase rate of brain myosin II is low, being < 30 nmol·min^{-1}·mg^{-1} even when maximally activated, and may be regulated through RLC phosphorylation [23–25]. Here, we have attempted to define the form of regulation of an individual isoform of brain myosin

Table 1
Actin-activated MgATPase activities of scallop hybrid myofibrils

Actin-activated MgATPase (μmol phosphate)/(min)(mg)			Sensitivity ratio	Reg/Ess
Calcium				
	(−)	(+)	(±)	
Light chain phosphorylation prior to readdition				
Desensitized scallop myofibrils	0.26	0.30	0.87	ND
Scallop RLC	0.07	0.36	0.19	ND
Non-phosphorylated rat brain RLC	0.25	0.27	0.93	0.86
Phosphorylated rat brain RLC	0.31	0.34	0.91	0.97
Light chain phosphorylation subsequent to hybrid formation				
Desensitized scallop myofibrils	0.16	0.14	1.14	ND
Scallop RLC	0.03	0.26	0.12	ND
Non-phosphorylated rat brain RLC	0.12	0.17	0.71	0.86
Phosphorylated rat brain RLC	0.14	0.15	0.93	0.96

The release of ^{32}P [21] was measured under standard conditions in the presence (+) (0.1 mM EGTA, 0.2 mM $CaCl_2$) or absence (−) (1.0 mM EGTA) of calcium. The ratio of the rates obtained in the absence of calcium to those obtained in its presence determines the sensitivity ratio (±). The Reg/Ess ratio indicates the level of light chain reuptake by the hybrid (maximum level, 1.0) and is measured by gel densitometry. ND: Not determined. Because of variation in activity between different preparations of scallop myofibrils, the results are tabulated in two groups with appropriate controls shown for comparison in both cases. In the upper group, phosphorylation of the RLC occurred on the isolated light chain prior to hybridization. In the lower group, phosphorylation of the RLC took place subsequent to hybridization.

R-LC DNA SEQUENCE AND AMINO ACID SEQUENCE COMPARISONS:

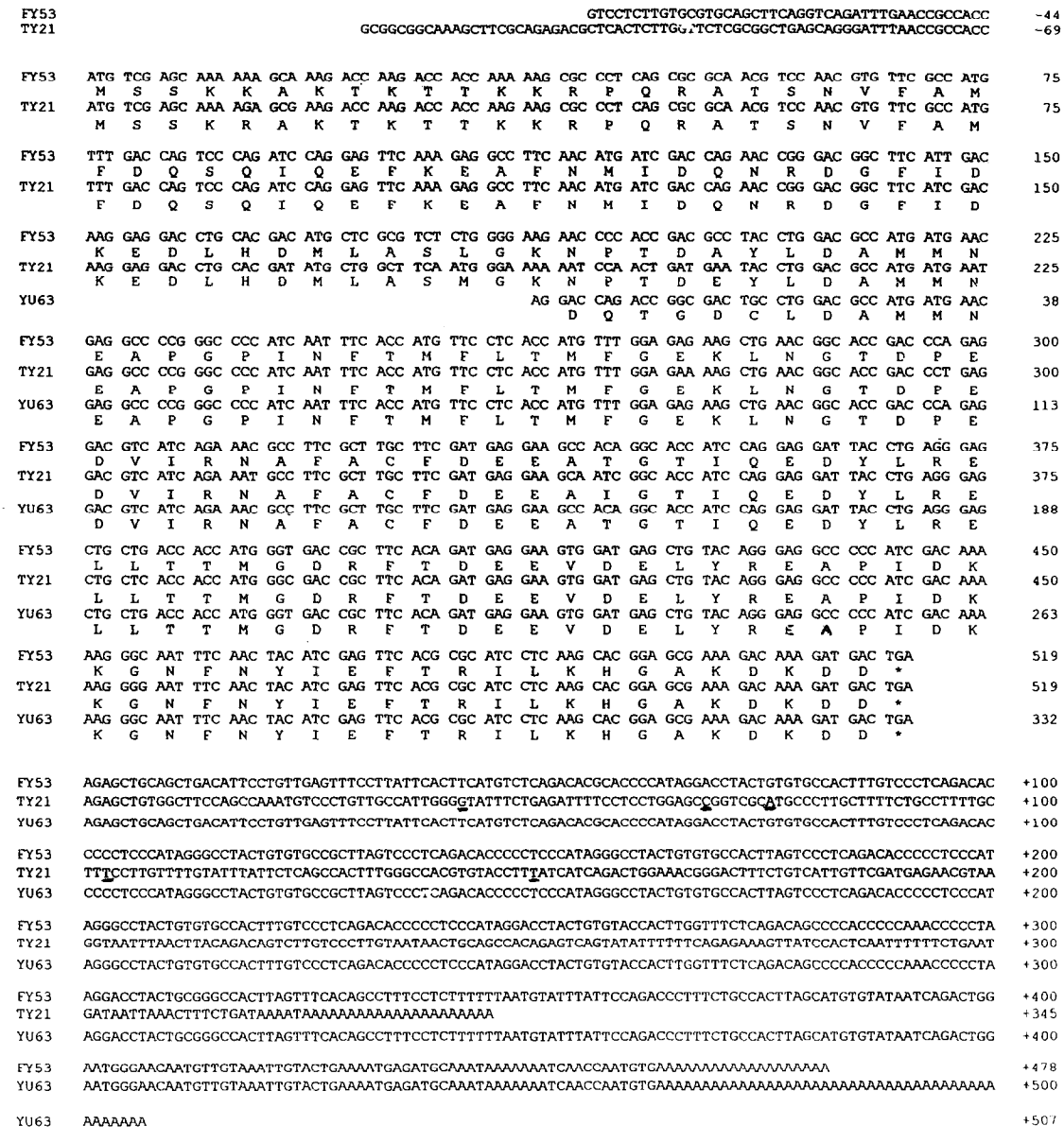

Fig. 1. Comparisons of cDNA and amino acid sequences of three myosin regulatory light chain isoforms found in rat brain. Sequences of the three different RLC cDNA sequences cloned here (FY53, TY21 and YU63) and their deduced amino acid sequences are displayed. Numbering: 5′-untranslated sequences are prefixed by a minus (−) sign; 3′ untranslated sequences are prefixed by a plus (+) sign. The single letter code for amino acids is displayed immediately below each codon. YU63 is unique. FY53 is identical to the RLC sequence described by Feinstein et al. [17] except that we have been able to extend their sequence further into the 5′-untranslated region and have completed the coding sequence (underlined). TY21 is similar to the RLC sequence described by Taubman et al. [22] except that a number of differences are seen when comparing TY21 with the revised version of the sequence submitted by these authors (Genbank Accession no. X05566). These differences are found in the 3′-untranslated region of the sequence and include insertions of G at +42, C at +69 and A at +76 and substitutions T for C at positions +103 and +150 (all underlined).

II from within that population by using scallop hybrid myosin as a test system [7,8,11]. In principle, our approach of initial cDNA library screening, use of the polymerase chain reaction [26] to generate a full length RLC cDNA coding sequence followed by incorporation into a prokaryotic expression vector, its overexpression in bacteria and introduction of the expressed product into a hybrid myosin, can be applied to any myosin isoform present in any complex tissue.

The lack of calcium-sensitive actin-activated MgATPase activity exhibited by the brain RLC (FY53) hybrid myosins (Fig. 2 and Table 1) was unexpected.

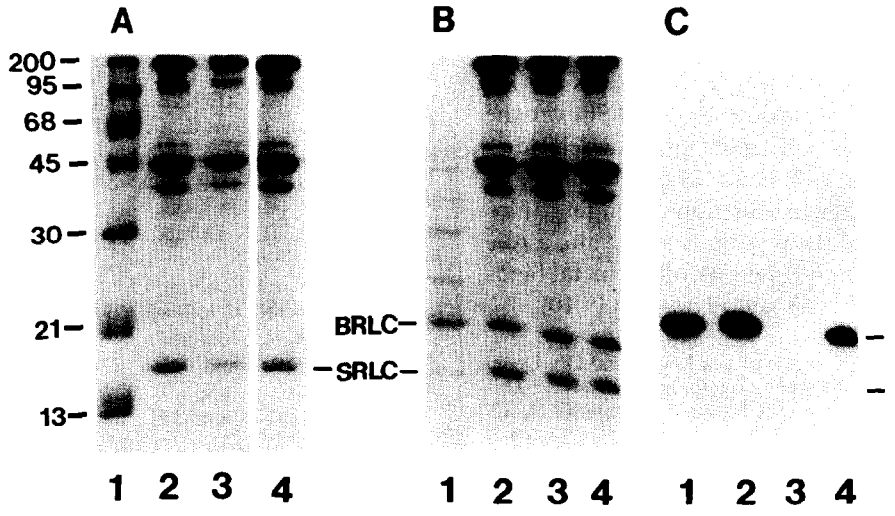

Fig. 2. Hybridization of expressed brain regulatory light chain with desensitized scallop myofibrils. (A,B) SDS/15% acrylamide gels stained with Fast green. (C) Autoradiograph of gel seen in B. Intact scallop myofibrils (A2) were desensitized (A3) then incubated with scallop RLC (control) (A4) or with rat brain RLC (FY53) (B,C, lanes 2–4) purified after overexpression in bacteria (B,C, lanes 1). Scallop hybrid myofibrils possess brain RLCs which are either non-phosphorylated (B,C, lanes 3), or were phosphorylated with MLCK either before (B,C, lanes 2) or after (B,C, lanes 4) hybridization. Loadings: 10 μg per lane. Lane A1: molecular weight standards. BRLC, brain regulatory light chain; SRLC, scallop regulatory light chain. Note that in these SDS/15% acrylamide gels, the scallop essential light chains run at the same location as the scallop regulatory light chain [4,5].

RLCs from both smooth and non-muscle myosins, capable of exerting control by derepression through RLC phosphorylation, have been shown to be capable of restoring calcium sensitivity to desensitized scallop myosin [7,12]. Previously, only RLCs from myosins found within thin-filament regulated muscles have produced calcium insensitive hybrids under these conditions [7–9]. The hybrid myosin rates seen here (Table 1) were elevated both in the presence and absence of calcium, similar to desensitized rates, as observed previously for pure vertebrate hybrids [7]; calcium insensitive inhibited rates, also reported for this type of hybrid [8,9], were not observed. RLC phosphorylation had no effect on these results, whether performed before, or after, hybrid formation (Table 1). It is unlikely that the absence of a blocked N terminus in the expressed FY53 RLC is responsible for disrupting regulation for full restoration of function has been achieved previously with scallop RLCs expressed in bacteria [10]. Indeed, disruption of either the divalent cation binding site [9,10], or the carboxyl terminus [10], is required in order to eliminate regulation while maintaining integrity of light chain binding. The possible significance of heavy chain phosphorylation as a regulatory mechanism in brain myosin [24,25,27,28], remains to be established but should not effect interpretation of the hybrid myosin results.

One interpretation would be that these results indicate the possible presence, in brain cells, of a myosin II isoform regulated by control proteins associated with the thin filament. While the presence of a thin filament controlled myosin in brain cells may appear unlikely, we note that other components associated with thin filament linked regulation, such as tropomyosin [31], troponin [32] and caldesmon [33], have also been found in brain.

Alternatively, it should also be borne in mind that the precise alignment of residues at the interface of the regulatory and essential light chains on the surface of myosin, required for the mechanism of allosteric control in a regulatory myosin, may not always be matched appropriately in hybrid molecules. The recently published structure of the regulatory domain from scallop myosin [29] indicates a key role for Gly^{117} in the RLC of that structure. Gly^{117} provides the two critical contact points between region III of the RLC and region I of the ELC within the regulatory myosin structure [29] and stabilizes the calcium-binding loop of the ELC. This glycine, together with flanking residues (MetGlyAsp) are conserved in the brain RLC sequences (Fig. 1; amino acid residues 130–132), raising the question as to why they are not capable of restoring functionality in these hybrid molecules (Table 1). It may be that the structure of the light chain interface is perturbed in these hybrids, thereby altering either the calcium binding domain itself or the allosteric relay mechanism. In this regard it may be noted that earlier hybrid studies detected a perturbation of function at the calcium specific site when pure hybrid myosins, formed using RLCs from regulatory myosins that operate through a phosphorylatory mechanism, exhibited calcium dependent regulation but with calcium binding curves shifted to higher free calcium levels as compared with intact scallop myosin [7].

In addition to extending the known cDNA sequence of one RLC isoform [17] further 5′, and defining a strain-variant of another RLC isoform [22], we also present

evidence here for the existence of a third RLC isoform in brain not described previously (Fig. 1). The major region of difference defined here, amino acids 63–68, appear to reside within a flexible loop [29,30] on the carboxyl side of the metal binding site, immediately adjacent to the F-helix of the E–F hand. This site may be of potential significance in the modulation of binding affinity between the RLC and the myosin heavy chain, a role which may help maintain the integrity of the metal binding site, crucial for inhibition and regulation [9,10].

Acknowledgements: We thank Professor Rhea Levine and Drs Deqin Li, Mark Miller, Anbin Mu and Weidong Sun for help and advice. This work was supported by a grant from the National Institutes of Health (AR 32858) to P.D.C.

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