

1 Creatine kinase is an alpha myosin heavy chain 3'UTR mRNA binding protein

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6 Abstract

7 Altered cardiac workload regulates the translation and localization of the α myosin heavy chain (α MyHC)
 8 messenger RNA through the 3' untranslated region (UTR) by protein–RNA interactions. We used the α MyHC
 9 3'UTR from neonatal rat heart tissue in a gel shift analysis to find RNA binding proteins. One was identified by
 10 microsequencing as creatine kinase, brain form B (CKBB). The affinity of its binding interaction was evaluated
 11 using sense and antisense α MyHC 3'UTR and 3' UTR probes from myosin isoforms of 2B and 2X skeletal muscle.
 12 Removal of calcium by the chelating agent EGTA had a potentiating effect on the formation of the CKBB/ α MyHC
 13 3'UTR complex *in vitro*. Varying the concentration of ATP (0.1–1 mM) also enhanced this interaction, suggesting
 14 that autophosphorylation of CKBB is taking place. Our novel finding that CKBB, an energy transduction enzyme,
 15 binds to the RNA of the 3'UTR of the faster ATP consuming α MyHC suggests a possible regulatory linkage
 16 between the metabolic state of the cell and myosin isoform expression.

17

18 Introduction

19 Mechanical, hemodynamic and hormonal stimuli regu-
 20 late gene expression and protein synthesis in the heart.
 21 For example, an increased demand for cardiac output
 22 initiates the hypertrophic response producing a greater
 23 muscle mass per nucleus (Hunter and Chien, 1999). The
 24 cellular response is achieved by a combination of
 25 changes to transcription, translation, synthesis, degra-
 26 dation and assembly of contractile proteins into the
 27 sarcomeres (McDermott and Morgan, 1989). Protein
 28 synthesis in general can be regulated by interactions
 29 between proteins and the untranslated regions (UTR) of
 30 the messenger RNA. Modulations of these interactions
 31 occur in many tissues and have been shown in muscle to
 32 control mRNA stability (Misquitta *et al.*, 2002), local-
 33 ization (Goldspink *et al.*, 1997; Nikcevic *et al.*, 1999,
 34 2000) or translation in physiological and pathophy-
 35 siological conditions (Ashley and Russell, 2000; Nussbaum
 36 *et al.*, 2002).

37 Altered workloads regulate both hypertrophy and the
 38 expression of contractile protein isoforms. The relation-
 39 ship between the velocity of the myosin cross bridge
 40 cycle and ATP consumption was first established by
 41 Barany (Barany, 1967). Small changes in the amount of
 42 the faster cycling ATPase of the α MyHC significantly
 43 augment myocyte power output (Herron and
 44 McDonald, 2002) because contractile velocity is largely
 45 determined by the proportion of the isoforms. Myosin
 46 isoforms can be altered by the functional demands of a

muscle cell (Eisenberg *et al.*, 1985) and also are regu- 47
 48 lated by the metabolic status of the animal induced by
 49 global events such as starvation that can be mimicked by
 50 deficiency of thyroid or phosphocreatine (Moerland
 51 *et al.*, 1989).

52 The α MyHC protein level decreases while the amount
 53 of α MyHC mRNA is increased when contractile activity
 54 is blocked in cultured neonatal heart cells (Samarel and
 55 Engelmann, 1991; Qi *et al.*, 1997). Here the decrease in
 56 protein synthesis is due to the block in translation of
 57 α MyHC via 3'UTR, while the increase in the α MyHC
 58 message is due to stabilization of the mRNA (Goldspink
 59 *et al.*, 1996, 1997). We used a luciferase reporter method
 60 to show that the 3'UTR of α MyHC regulates translation
 61 when calcium cycling is attenuated, regardless of loca-
 62 tion of the message and suggested a possible mechanism
 63 might be due to the binding of proteins to the 3'UTR
 64 (Nikcevic *et al.*, 2000). Therefore, the aim of this study
 65 is to identify and characterize such hypothetical binding
 66 proteins, determine their binding affinities to the
 67 α MyHC-3'UTR and explore possible mechanisms for
 68 regulation of the protein–RNA interactions.

69 Materials and methods

70 Cardiac tissue and cells

71 Hearts of the 1–2-day old Sprague–Dawley rats were
 72 used to culture cells or to prepare the tissue homogenate
 73 as previously described (Nikcevic *et al.*, 2000). Ap-
 74 proved animal handling protocols were followed at all
 75 times. Cardiomyocytes were plated on laminin-coated

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76 dishes at high density (1.4×10^3 cells/mm ²). The culture	were analyzed by agarose gel electrophoresis and their	129
77 medium was a 1:1 mixture of Modified Dulbecco's	concentrations were determined by spectrophotometry.	130
78 culture medium (Sigma, St. Louis, MO) and Ham's F-12		
79 nutrient mixture, supplemented with thyroxin (0.5 nM).		
80 <i>Preparation of cytoplasmic extracts</i>		
81 Cultured cells were washed twice with phosphate buf-	Protein-RNA interactions were determined from lysing	132
82 fered saline (PBS), and scraped in 200 μ l of cell lysis	10–20 μ g of total protein from the whole heart or	133
83 buffer (CLB) as described previously (Nikcevic <i>et al.</i> ,	cytoplasmic extracts from cultured cells using 40U of	134
84 2000). Freshly dissected neonatal hearts were washed	RNAase inhibitor (Roche Diagnostics GmbH,	135
85 twice in PBS, trimmed to remove atria, minced in 1:15	Mannheim, Germany). This mixture was incubated with	136
86 volume of cold CLB and homogenized using an Omni-	³² P labeled 3'UTR probe ($\sim 5 \times 10^4$ cpm) for 30 min at	137
87 mixer (Omni International) at 3000 rpm with additional	room temperature in a 20 μ l reaction volume. Heparin	138
88 sonication. The cytosolic fractions of tissue homogenate	(5 μ g/ μ l) was added to the reaction for the last 10 min in	139
89 and cell lysate were obtained by centrifuging samples for	order to reduce nonspecific binding. After hybridization,	140
90 1 h at 40,000 rpm with a Beckman Ti50 rotor at +4°C.	the RNA-protein complexes were subjected to nonde-	141
91 The fractions were the supernatant with the cytosolic	naturing electrophoresis on 5% polyacrylamide gels in	142
92 proteins and the pellet with membrane, organelle and	1xTBE buffer, (1 M Tris, 0.9 M Boric acid, 0.01 M	143
93 cytoskeleton proteins. The protein concentration of each	EDTA), and analyzed using a phosphoimager (Molec-	144
94 sample was determined by the Bradford method	ular Dynamics Inc.)	145
95 (Bio-Rad, Hercules, CA).		
96 <i>Pharmacological treatments</i>	<i>Protein elution, peptide sequencing and amino acid</i>	146
97 Pharmacological treatment changed the contractile	<i>sequence analysis</i>	147
98 activity of cardiac cells and induced translational block		
99 of α MyHC mRNA as previously shown by our group	Proteins were isolated from the RNA-protein complex	148
100 (Nikcevic <i>et al.</i> , 2000). The mechanism by which change	formed in a preparative gel shift assay. Briefly, mixture	149
101 in contractile activity induced translational block might	of ³² P labeled and unlabeled 3'UTR probes were	150
102 be due to an altered affinity of RNA binding proteins. In	incubated at room temperature with 400–500 μ g of	151
103 order to test that notion, contractility of cardiomyocytes	protein from the cytoplasmic extract of whole neonatal	152
104 in culture was blocked with 10 μ M verapamil (Sigma,	rat heart. The complex was electrophoretically separated	153
105 St. Louis, MO) or the strength and frequency of beating	on a large nondenaturing 5% acrylamide gel. After	154
106 was increased with 10 μ M L-isoproterenol HCl (Sigma,	electrophoresis, the wrapped gel was placed on a	155
107 St. Louis, MO) (Goldspink <i>et al.</i> , 1997). Prior to	phosphoimage screen and exposed for 2–24 h. Phos-	156
108 pharmacological treatments, cells were placed in serum	phoimage analysis was performed using a Phosphoim-	157
109 free medium for 24 h. Following 6 h of drug treatment	ager (Molecular Dynamics). The gel image was printed	158
110 cells were scraped and processed for subcellular protein	and aligned with the gel to identify the position of the	159
111 fractionation. The control cardiomyocytes were main-	retarded complex, which was then excised and minced.	160
112 tained identically except for the drug treatment.	Proteins were eluted from the gel using Bio-Rad	161
113 <i>Plasmid constructs and in vitro transcription</i>	Electro-Eluter model 422, (Bio-Rad, Hercules, CA) in	162
114 The full-length rat α MyHC-3'UTR cDNA, 100 bases	1xTris/borate/EDTA elution buffer at 8–10 mA per	163
115 long (5822–5921) was subcloned in the pCRII vector.	glass tube. Eluted proteins were collected and concen-	164
116 MaxiScript TM SP6/T7 <i>in vitro</i> Transcription kit	trated using a Millipore Ultrafree-0.5 centrifugal filter	165
117 (Ambion, Austin, TX) that was used to generate sense	device. Samples were then separated on precast 4–15%	166
118 or antisense RNA probes, labeled with either ³² P or	gradient SDS polyacrylamide gels (Bio-Rad, Hercules,	167
119 biotin. The probes for the fast skeletal myosin heavy	CA) and stained with Coomassie blue. Bands were	168
120 chain isoforms were kindly provided by Prof. G.	excised and sequenced at the Harvard Microchemistry	169
121 Goldspink, Royal Free, London, UK. For synthesis of	Laboratory, Cambridge, MA, using micro-capillary	170
122 either ³² P or biotin labeled RNA transcripts, we used	reverse phase high-pressure liquid chromatography	171
123 2X-MyHC (432–643) and 2B-MyHC cDNA (containing	and nano-electrospray tandem spectrometry (μ LC/MS/	172
124 the complete 3'UTR and coding sequence 1–148),	MS) on a Finnigan LCQ DECA quadrupole ion trap	173
125 subcloned in pGEM-4Z. The unincorporated nucleo-	mass spectrometer. The MS/MS spectra obtained were	174
126 tides were removed using sephadex spin columns	then correlated with known sequences using the Sequest	175
127 (ProbeQuant G-50 Micro Columns, Amersham	algorithm.	176
128 Pharmacia Biotech, Piscataway, NJ). The transcripts	<i>Specific RNA affinity binding</i>	177
	Specific RNA-protein binding affinity was monitored	178
	using beads with streptavidin attached (BioMag, Poly-	179
	sciences Inc., Warrington, PA) and a magnetic separa-	180
	tor. The magnetic beads, 40 μ l, were incubated with	181

182	0.2 µg of the biotinylated αMyHC-3'UTR RNA tran-	239
183	script at room temperature for 15 min to allow the	240
184	probe to attach to streptavidin. The beads were mag-	241
185	netically separated and washed with CLB to eliminate	242
186	the unbound probe. Protein (10–50 µg) taken from the	243
187	cytosolic extract of hearts from neonatal or adult rats	
188	was incubated with probe-bound beads in CLB con-	
189	taining 4U of RNase inhibitor (Roche Diagnostics	
190	GmbH, Mannheim, Germany) for 2 h at 4°C.	
191	CKBB is highly conserved throughout evolution and	
192	the rat sequence is over 90% identical with the human	
193	CKBB isoform allowing use of a commercially available	
194	recombinant human molecule to test binding specificity	
195	in rat studies (Research Diagnostics Inc., Flanders, NJ).	
196	Other commercially available purified proteins were also	
197	used in this assay; rabbit heart lactic dehydrogenase	
198	(LDH) (Sigma, St. Louis, MO), yeast phosphoglycerate	
199	Kinase (PGK) (Molecular Probes, Eugene, OR) and	
200	human fatty acid binding protein (FABP) (Research	
201	Diagnostics Inc., Flanders, NJ).	
202	Nonspecific binding was controlled by adding RNA	
203	competitors such as heparin (5 µg/µl) and/or yeast	
204	tRNA (0.2 µg/µl) into the reaction during the last	
205	30 min. The beads were then extensively washed with	
206	CLB. The bound proteins were eluted and separated on	
207	precast 4–15% gradient SDS polyacrylamide gels	
208	(Bio-Rad, Hercules, CA). Proteins were then either	
209	stained with silver or Coomassie blue, or transferred	
210	directly onto a nitrocellulose membrane.	
211	To test the effect of divalent cations on the protein	
212	binding affinity with αMyHC-3'UTR, the heart cell	
213	extracts were incubated in the presence of 10 mM	
214	divalent cation chelators, EDTA or EGTA.	
215	To test the modulatory effect of ATP on RNA	
216	binding, an affinity-binding assay was performed using	
217	recombinant human CKBB preincubated in CLB con-	
218	taining different concentrations of ATP (0.1 and 1 mM)	
219	for 1 h at 37°C.	
220	<i>Western blotting</i>	
221	Protein samples were prepared in Laemmli buffer and	
222	separated on 4–15% gradient SDS-polyacrylamide gels.	
223	They were transferred and stored overnight in 1xTris/	
224	glycine buffer pH 8.6 on Hybond nitrocellulose mem-	
225	branes (Amersham Pharmacia Biotech, Piscataway,	
226	NJ). In order to block nonspecific reactions, membranes	
227	were incubated in 10% dry milk in 0.01 M PBS and	
228	0.2% Tween 20.	
229	Membranes were probed with different primary anti-	
230	bodies using 1:250 to 1:1000 dilutions (polyclonal rabbit	
231	antihuman CKBB, OEM Concepts, Toms River, NJ;	
232	monoclonal mouse antirabbit LDH, Sigma, St. Louis,	
233	MO; monoclonal mouse antihuman FABP, Research	
234	Diagnostics Inc., Flanders, NJ; polyclonal mouse	
235	anti yeast PGK, Molecular Probes, Eugene, OR). After	
236	brief washing, membranes were incubated with an	
237	anti-mouse or anti-rabbit secondary antibody,	
238	conjugated with horseradish peroxidase (Research	
	Diagnostics Inc., Flanders, NJ). An ECL kit (Amer-	239
	sham Pharmacia Biotech, Piscataway, NJ) was used for	240
	signal detection. Exposed films were scanned and the	241
	signals were quantified using Image QuANT software	242
	(version 4.2a).	243
	<i>Autophosphorylation assay</i>	244
	Autophosphorylation of recombinant human CKBB	245
	was performed as described previously (Quest <i>et al.</i> ,	246
	1990; Stolz <i>et al.</i> , 2002). Briefly, recombinant human	247
	CKBB was incubated in an autophosphorylation buffer	248
	(AP buffer) (100 mM glycylglycine, 2 mM magnesium	249
	acetate, 0.5 mM EDTA, 2 mM dithiothreitol (DTT), pH	250
	8.4) at a concentration of 0.5 mg/ml containing 50 µCi	251
	[γ- ³² P] ATP (10 mCi/ml) for 2 h at 37°C. Unreacted	252
	γ- ³² P ATP was removed by size-exclusion chromatogra-	
	phy on Sephadex G-50 columns and the proteins were	254
	separated on precast 4–15% gradient SDS polyacryl-	255
	amide gels (Bio-Rad, Hercules, CA). Gels were exposed	256
	to the intensifying phospho-storage screen and analyzed	257
	using a phosphorimager (Molecular Dynamics Inc.).	258
	<i>Two-dimensional gel electrophoresis</i>	259
	Isoelectric focusing (IEF) was performed using Bio-Rad	260
	Protean IEF cell as per manufacturer instructions	261
	(Bio-Rad, Hercules, CA). IPG strips (11 cm, pH 5–8) were	262
	rehydrated overnight in the standard rehydration buffer	263
	(8 M urea, 2 M thiourea, 4% CHAPS w/v, 2 mM EDTA	264
	pH8, 250 mM DTT, 2 mM MTBP, ampholytes 3–10) together	265
	with samples. Following 17 h of rehydration IPG strips were	266
	transferred in the IEF cell and overlaid with mineral oil to	267
	prevent dehydration. Samples were run for 5.5 h and the	268
	voltage applied according to the program: 20 min 250 V,	269
	2.5 h 8000 V and continued for the total of 20000 V h. Prior	270
	to running the second dimension, gel strips were equilibrated	271
	in a buffer that contains SDS (6 M urea, 0.375 M Tris pH 8.8,	272
	2% SDS, 20% glycerol and 2% w/v DTT in buffer 1, or in	273
	buffer 2–2.5 w/v iodoacetamide) in two 15 min steps.	274
	Horizontal electrophoresis was performed using a 12% T	275
	resolving gel in Bio-Rad criterion electrophoresis cell (Bio-	276
	Rad, Hercules, CA) for 60 min at 200 V constant voltage.	277
	Following SDS-PAGE, gels were stained with Sypro ruby	278
	fluorescent protein gel stain (Molecular Probes, Eugene,	279
	OR) as per manufacturer instructions and scanned on a Bio-	280
	Rad Molecular Imager® FX Pro Plus™. To identify the	281
	position of the CKBB spots on the gel, proteins were	282
	transferred on a PDF membrane and Western blot was	283
	performed as previously described.	284
	Results	285
	<i>Protein binding to the MyHC</i>	286
	Proteins from the cytosolic protein extracts bound to	287
	the αMyHC-3'UTR, as shown in a representative	288
	nondenaturing polyacrylamide gel (Figure 1a). Protein	289

290 concentration titration experiments showed that 10 μ g
 291 was sufficient for gel shift assays (data not shown) so
 292 10 μ g was loaded in all lanes. We found that almost all
 293 of the probe from the whole heart was bound despite
 294 the use of the nonspecific competitor heparin to
 295 eliminate nonspecific binding. Although there was a
 296 shift in electrophoretic mobility, silver staining showed
 297 that the same molecular weight proteins were involved
 298 in RNA binding in both cytosolic extracts from the
 299 whole heart and from cells cultured from neonatal rat
 300 (Figure 1b).

301 Identification and sequencing of bound proteins

302 The α MyHC-3'UTR complexes yielded distinct protein
 303 bands (Figure 1b), that were then eluted from the gel.
 304 Proteins were sequenced and related to their respective
 305 gel bands and actual molecular weights (Table 1). The
 306 proteins were identified as creatine kinase BB isoform
 307 and phosphoglycerate kinase that comigrate within the
 308 same 49 kD band, lactic dehydrogenase at 37 kD, and
 309 fatty acid binding protein at 15 kD. A band at 70 kD

Table 1. A list of proteins involved in complex formation with α MyHC-3'UTR, identified by sequencing

Gel band ~MW (kD)	Protein	Protein MW (kD)	NCBI accession #	% of protein sequenced
49	Phosphoglycerate kinase, EC 2.7.2.3.	44.5	A33792	33
49	creatine kinase B, EC 2.7.3.2.	42.7	KIRTCB	37
37	Lactate dehydrogenase B, EC 1.1.1.27	36	P42123	49
15	Fatty acid binding protein	14.7	A27452	67

identified as bovine serum albumin, a very common
 contaminant probably carried forward from the culture
 media via the reused eluter membranes.

RNA affinity binding analysis

To confirm microsequencing results with an independent
 technique, RNA affinity binding analysis was
 performed using biotin labeled MyHC-3'UTR bound
 to streptavidin coated magnetic beads. The bands and
 molecular weights were similar to the proteins eluted
 from the gel shift analysis (data not shown) and Western
 blots confirmed that they corresponded to proteins
 identified by sequencing.

The pure recombinant protein of human and the
 CKBB isolated from the whole heart were seen as a
 49 kD band (Figure 2a). Proteins were incubated with
 the probe and separated on a gel under denaturing
 conditions before western blotting. The position of the
 bands were retarded to 60–63 kD suggesting that the
 protein-3'UTR complex might still be bound (Figure
 2a).

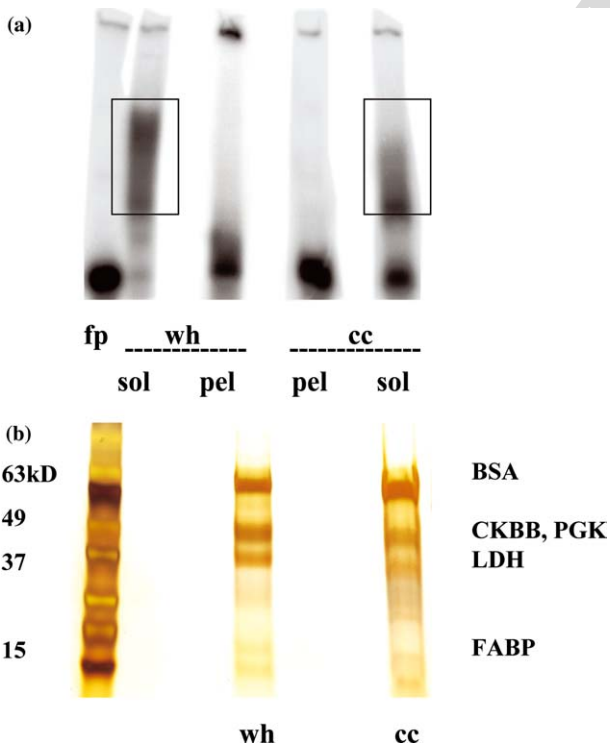


Fig. 1. (a) α MyHC 3'UTR complex formation with heart cell proteins. RNA gel shift analysis on a nondenaturing polyacrylamide gel showing complexes formed between P^{32} labeled α MyHC 3'UTR probe and proteins. The free probe lane (fp) has P^{32} - α MyHC 3'UTR but contains no protein. RNA retardation by protein binding are whole heart proteins (wh) from neonatal rat, cytosolic proteins from cultured cardio-myocytes (cc). After centrifugation at 40,000 rpm, proteins are fractionated into cytosolic (sol) and pellet (pel). Note that smeared retarded complexes with sol proteins in the same position (boxed areas) from both wh and cc. (b) Silver staining of the RNA binding proteins eluted out of the complex shown in boxed regions of panel (a) and labeled according to later sequencing data as CKBB, lactic dehydrogenase (LDH), FABP and contamination by bovine serum albumin (BSA). Left lane, molecular weight size marker (Invitrogen).

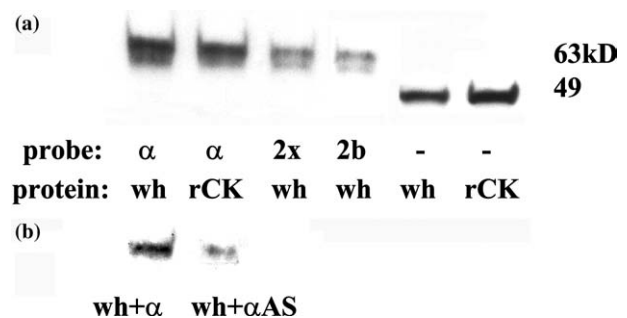


Fig. 2. RNA binding specificity of creatine kinase brain isoform B (CKBB) with different RNA probes. Western blotting for CKBB follows an affinity-binding assay for RNA. (a and b) Probes are α , α MyHC 3'UTR (full length 3'UTR); 2X, rat skeletal isoform 2X-MyHC 3'UTR (432–643); rat skeletal isoform 2B-MyHC 3'UTR (1–148); α AS, antisense α MyHC 3'UTR. Proteins are wh, cytosolic fraction from whole heart of neonatal rat; rCK, recombinant human CKBB. (c) Quantified optical density from α MyHC 3'UTR sense (α) or antisense (α AS) probes retarded by neonatal rat heart cytosolic proteins (wh), ($n = 3$).

330 However, other proteins were not so readily con-
 331 firmed. LDH (purified from whole rat or rabbit heart)
 332 did not bind directly to the α MyHC-3'UTR and
 333 interaction through coupling was also ruled out even
 334 though the antibody was sensitive enough to detect the
 335 presence of LDH (Figure 3a). Similar binding assays
 336 using purified human FABP and yeast PGK showed
 337 that the purified proteins were able to form a complex
 338 with α MyHC-3'UTR but the respective proteins in the
 339 complex were not detected with cytosolic extracts from
 340 whole rat heart, at least within the range of the protein
 341 amounts we used (50–100 μ g) (Figure 3b and c). It is
 342 possible that all these proteins co-migrate with the
 343 complex or are loosely associated with the complex
 344 when separated under the native conditions that would
 345 not necessarily be seen under denaturing conditions
 346 found in the RNA affinity-binding assay.

347 Affinity of the α MyHC 3'UTR–protein interaction

348 In our previous study (Nikcevic *et al.*, 2000), we did
 349 competition studies with a cold probe and confirmed the
 350 binding specificity to the sense but not the antisense
 351 strand of α MyHC-3'UTR. Here, we extended those
 352 studies in a set of RNA affinity binding experiments
 353 using the skeletal muscle MyHC-3'UTR isoforms 2X

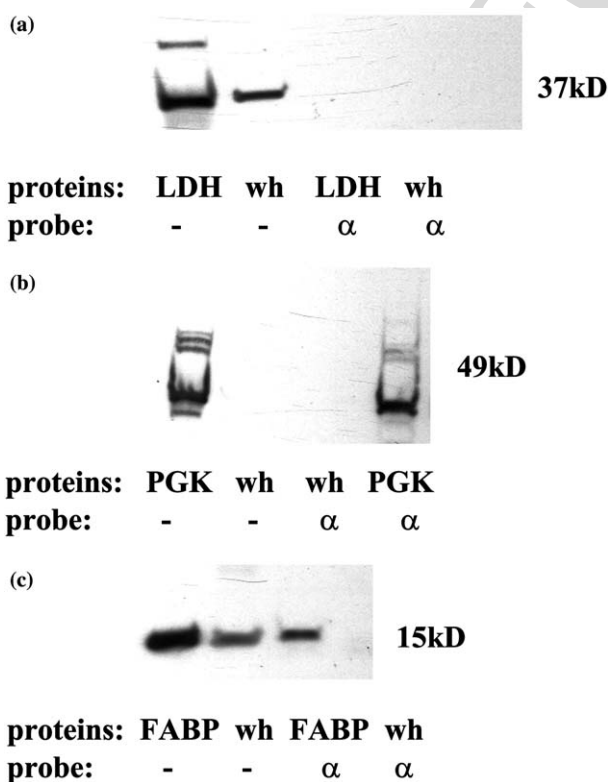


Fig. 3. Binding assay for LDH, PGK and FABP from rat heart and α MyHC 3'UTR. RNA affinity binding assay followed by Western blotting shows (a) LDH from rat heart (50 μ g) or purified rabbit LDH (5 μ g) do not bind 3'UTR probe; (b) PGK of the heart does not bind but has a small detectable interaction with purified yeast PGK (5 μ g); (c) rat heart FABP does not bind but 5 μ g of purified human FABP slightly interacts with α MyHC 3'UTR.

and 2B, followed by Western blotting. The skeletal
 isoforms bound more weakly to CKBB than to
 α MyHC-3'UTR (Figure 2a). The binding appeared to
 be strong since the electrophoretic mobility shift was
 seen even after separating proteins under denaturing
 conditions when compared with free whole heart or
 recombinant CKBB. Taken together, our results suggest
 that CKBB preferentially bind to the α isoform.

Special affinity of the binding protein for the
 sense form was demonstrated using an antisense
 α MyHC-3'UTR transcript. A very light band was seen
 using the antisense α MyHC-3'UTR probe (Figure 2b)
 that was quantified (Figure 2c).

Effect of divalent cation removal on RNA binding affinity

Removal of Ca^{++} and Mg^{++} by buffering with EDTA
 enhanced the binding affinity of CKBB to α MyHC

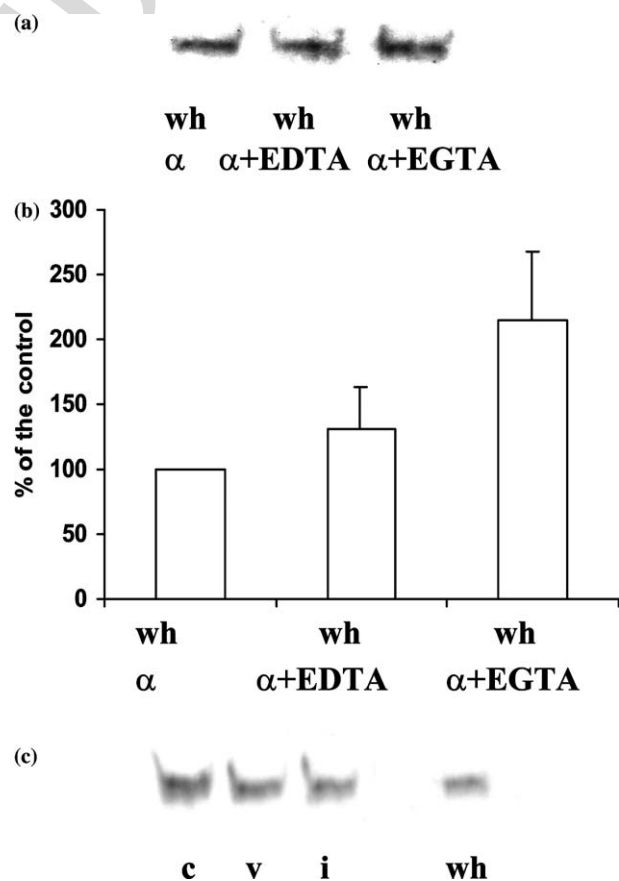


Fig. 4. Withdrawal of calcium cation affects CKBB complex formation. Western blotting for CKBB follows an RNA affinity-binding assay. (a) Neonatal heart cytosolic proteins and probe: α , α MyHC 3'UTR; α + EDTA, α MyHC 3'UTR in the presence of EDTA; α + EGTA, α MyHC 3'UTR in the presence of EGTA. (b) Histogram shows the relative change in the amount of bound CKBB to α MyHC 3'UTR probe upon divalent cation removal expressed as a percentage of the control reaction between heart cytosolic proteins and α MyHC 3'UTR (α); ($n = 4$ with SE bar, $P = 0.05$). (c) RNA affinity binding assay with bound CKBB detected by Western blotting. CKBB binding to α MyHC-3'UTR in cardiomyocyte cell cytosolic extract after 6 h treatment with verapamil (v), isoproterenol (i) or control (c). Whole neonatal rat heart cytosolic extract (wh) served as a positive control.

370 3'UTR mRNA (Figure 4a). Quantification by OD
371 showed that the binding affinity of the cation-depleted
372 samples was 20% more than when cations were present
373 ($131 \pm 31\%$) (Figure 4b). Removal of calcium by
374 EGTA more than doubled the binding affinity of CKBB
375 ($214 \pm 54\%$), compared to the control of 100% ($n = 4$,
376 $P = 0.05$). Thus, removal of Ca^{++} had a potentiating
377 effect on the formation of the protein/ α MyHC-3'UTR
378 complex *in vitro* (Figure 4b).

379 Effect of verapamil and isoproterenol on α MyHC-3'UTR/ 380 protein interactions

381 We had previously reported that 6 h verapamil treat-
382 ment induced a reduction in α MyHC 3'UTR mRNA
383 translation (Nikcevic *et al.*, 2000) but did not determine
384 the nature of this protein complex. Here, while we found
385 that CKBB was present in untreated cardiac cells as well
386 as contractile-arrested cells, we did not find a significant
387 difference in the band intensity between extracts of
388 control and drug-treated cells (Figure 4c). This finding
389 suggested that our translational block hypothesis could
390 not be explained by the differential binding affinity in
391 verapamil treated cells. However, our methodology
392 might not be sensitive enough to pick up such subtle
393 changes in the affinity of binding.

394 The effect of ATP on RNA binding

395 The recombinant human CKBB RNA binding capacity
396 was modulated by preincubation with ATP. Incubation
397 of CKBB with different concentrations of ATP within
398 and below the physiological ATP concentration range
399 (0.1–1 mM) affected the binding with α MyHC-3'UTR
400 (Figure 5a). The effect was dose dependent with the
401 highest ATP concentration used (1 mM) causing the
402 highest CKBB binding (Figure 5a).

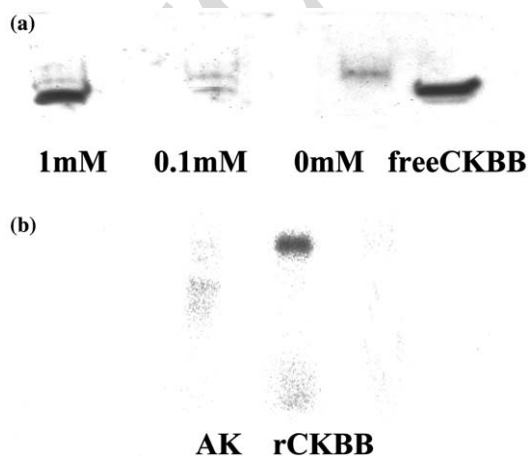


Fig. 5. (a) Autophosphorylation of CKBB enhances complex formation. Preincubation of recombinant human CKBB protein (recCKBB, 0.25 μ g) with different concentrations of ATP stimulates CKBB α MyHC-3'UTR binding; concentrations of ATP are 1, 0.1 mM, or 0 mM ATP. (b) Autophosphorylation of recCKBB in a presence of γ - P^{32} ATP. (AK), adenylate kinase (EC 2.7.4.3) was used as a control.

Autophosphorylation of CKBB

403

An autophosphorylation assay was performed in a
404 presence of radioactive γ - P^{32} ATP to investigate the
405 potential role of autophosphorylation in the regulation
406 of the RNA binding activity of CKBB. Phosphoimage
407 analysis confirmed that autophosphorylation of recom-
408 binant human CKBB was possible (Figure 5b) since
409 radioactivity was detected only in the CKBB lane but
410 not in control with the enzyme adenylate kinase.
411

Two-dimensional polyacrylamide gel and blot analysis

412

Neonatal rat ventricular tissue samples were analyzed
413 using 2D gel electrophoresis. Computational pI for the
414 rat CKBB is 5.33 therefore we used IPG gel strips with
415 pH range 5–8, to achieve separation. Many proteins
416 were resolved in this pI range (Figure 6a). Western
417 blotting was performed to identify the position of the
418 CKBB on the gel. CKBB blot images showed spots
419 around the expected size range of 45–49 kD (Figure 6b).
420 The protein retardation toward the acidic side of the gel
421 might be an indication of post-translational modifica-
422 tion by phosphorylation.
423

Discussion

424

We found that the 3'UTR of the α MyHC message
425 formed a complex with several proteins related to energy
426 metabolism; namely creatine kinase B, lactic dehydroge-
427 nase B, phosphoglycerate kinase, and fatty acid binding
428 protein. Of these, we focused on CKBB that showed a
429 higher affinity for the sense than either the antisense
430 3'UTR α MyHC or the 2X and 2B myosin heavy chain
431

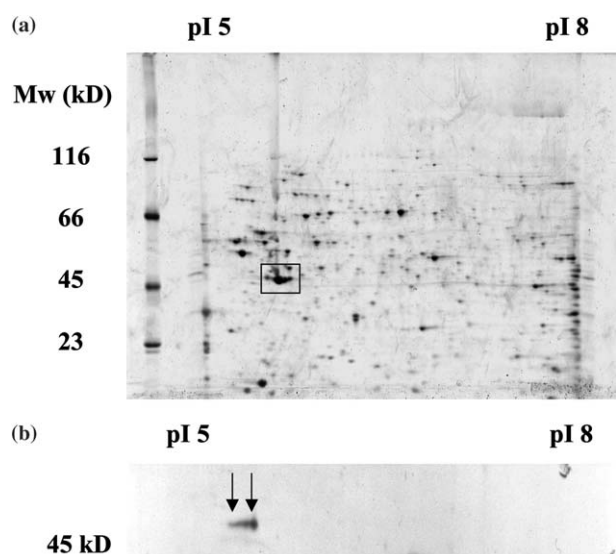


Fig. 6. (a) 2D gel electrophoresis. Image of proteins from neonatal rat heart stained with Sypro Ruby. The boxed spot corresponds to CKBB; (b) Western blot of the heart CKBB resolved on 2D gel. Arrows point to several spots with slightly different pI indicating possible phosphorylation.

432 isoforms of skeletal muscle. Also, cations and ATP
433 affected the binding affinity of CKBB to the 3'UTR
434 α MyHC. This is the first report of CKBB interacting with
435 a contractile protein although, interestingly, during
436 osteoblastic differentiation, CKBB protein synthesis is
437 regulated by a complex formed between its own 3'UTR
438 and cytosolic proteins (Ch'ng and Ibrahim, 1994).

439 The CKBB is the predominant isoform in embryonic
440 heart, down regulated as the animal develops but can be
441 re-expressed in adult heart in response to stimuli
442 producing ischemia, hypertrophy or heart failure
443 (Ingwall, 1993; Ritchie, 1996). This is a similar re-
444 expression pattern for the fetal contractile isoforms,
445 perhaps suggesting a coupling between metabolic rates
446 and contractile activity patterns. The myosin ATPase
447 cycle coordinates with the CK cycle to provide optimal
448 conditions for actin-myosin interaction (Masataka
449 *et al.*, 1996). Functional interactions between CK iso-
450 forms and contraction are further suggested given their
451 myofibrillar compartmentalization. CKBB is localized
452 to the Z-band in chicken heart (Wallimann *et al.*, 1977)
453 and to the I band of skinned skeletal muscle fibers. The
454 BB isoform may produce ATP more effectively than
455 MM due to its higher affinity for ADP (Ingwall, 1993).

456 Different concentrations of ATP altered the RNA
457 binding affinities of recombinant CKBB, implicating a
458 role for ATP in modulation of translational control of
459 α MyHC. Phosphorylation by other nucleotides was not
460 studied and the possibility of their involvement remains
461 for future studies. An informatics search (ExPasy web
462 site) for post-translational modifications sites within the
463 CKBB sequence indicated the possibility of phosphor-
464 ylation of several serine and tyrosine residues. Confir-
465 mation of the phosphorylation of the chicken CKBB has
466 been reported (Quest *et al.*, 1990; Stolz *et al.*, 2002). Our
467 two dimension electrophoresis results also indicated a
468 possibility of CKBB phosphorylation from whole neo-
469 natal rat heart tissue. The autophosphorylation of
470 CKBB may depend on pH and the available levels of
471 ATP, ADP and creatine phosphate important for the
472 primary function of "energy shuttling" in a cell. Indeed,
473 the AMP-activated protein kinase inhibits CKMM by
474 phosphorylation *in vitro* and in differentiated muscle
475 cells under the conditions in which PCr/Cr and ATP/
476 ADP levels fall (Ponticos *et al.*, 1998). Correlation
477 between the activity of CK and AMPK in the heart
478 was found to be positive, complex and nonlinear
479 (Ingwall, 2002). Therefore, we might speculate that
480 autophosphorylation and/or phosphorylation modu-
481 lates the binding affinity of CKBB to α MyHC-3'UTR
482 *in vivo* thus affecting translation.

483 A reduction in translation of α MyHC was found when
484 verapamil prevented calcium from initiating the cross
485 bridge cycle (Samarel and Engelmann, 1991; Nikcevic
486 *et al.*, 2000) with the resulting decline in ATP consump-
487 tion. Here we confirmed that Ca^{2+} depletion directly
488 enhanced the CKBB binding affinity to α MyHC 3'UTR
489 *in vitro*. However, we note that CKBB is not itself a Ca^{2+}
490 binding protein so that a simple calcium regulatory

mechanism is unlikely. An indirect mechanism might
491 occur, for example, it is known that verapamil can
492 abolish site specific phosphorylation of phospholamban
493 in the rat hearts thus affecting SR Ca^{2+} ATPase activity
494 and consequently contractility (Kuschel *et al.*, 1999). We
495 may speculate that CKBB phosphorylation in the rat
496 cardiac cell can also be indirectly affected by verapamil
497 induced Ca^{2+} depletion leading to altered binding of the
498 α MyHC 3'UTR and subsequently of translation.
500 Although CKBB is the protein that actually binds to
501 the mRNA, taking all our findings together we suggest
502 that the alterations in the cellular calcium level might
503 trigger some secondary event in the cytosol when the
504 CKBB phosphorylation state is conducive for action.
505 The mechanism is obviously not simple and might well
506 involve several proteins in a complex or cascade fashion
507 to control myosin isoform translation.

508 There is growing evidence that metabolic enzymes may
509 act as multifunctional proteins performing diverse roles
510 in the cell including translational control (Nikcevic *et al.*,
511 2000; Kiri and Goldspink, 2002), and replication and
512 repair of DNA (Popanda *et al.*, 1998; Nagy *et al.*, 2000).
513 Several examples of binding by metabolic enzymes to
514 RNA are known, including LDH, PGK, GAPDH,
515 aldolase and mitochondrial aconitase (the iron-response
516 element binding-protein) (Kaptain *et al.*, 1991; Sirover,
517 1996; Nagy *et al.*, 2000; Kiri and Goldspink, 2002). The
518 role of the rat heart FABP in translational control has
519 also been reported (Zimmerman and Veerkamp, 1998).
520 Functional association of the CKMM isoform with
521 glycolytic enzymes is also very well established at the
522 sarcomere level (Kraft *et al.*, 2000) and in the sarcoplasm-
523 ic reticulum membranes (Xu and Becker, 1998) sup-
524 porting the hypothesis that locally generated ATP by
525 glycolytic enzymes is coupled to the site of energy
526 utilization. Our findings indicate that LDH, PGK and
527 FABP might also be involved in interaction with α MyHC
528 3'UTR but not directly as binding proteins. They might
529 have a more supportive role than a loosely associated
530 complex with CKBB, further providing the link between
531 different pathways of energetic metabolism and contrac-
532 tile protein synthesis. Therefore, metabolism may
533 co-ordinate numerous subcellular processes via nucleo-
534 tide interactions in both the nucleus and the cytoplasm.

535 Using ExPasy proteomics server to scan for motifs
536 and patterns within the CKBB sequence we did not find
537 any known consensus RNA binding domains. None-
538 theless, the secondary structure of α MyHC 3'UTR as
539 projected (mFold 2.3 web server, Zuker, 2003) reveals a
540 hairpin loop with several bulges that might be potential
541 sites for protein binding. We did not explore the cis
542 sequences to which CKBB binds and UTRscan (a UTR
543 sequence analysis web tool) did not find any known
544 consensus RNA binding sequence. Future studies dis-
545 secting the 3'UTR region of α MyHC will be needed to
546 address the exact CKBB binding domain.

547 In this report, CKBB is shown for the first time to
548 have an RNA binding function in cardiac muscle cells.
549 We suggest that CKBB phosphorylation is part of a

550 complex mechanism for the regulation of RNA binding
551 and translational control of the α MyHC. Since CKBB is
552 an "energy shuttling" enzyme, it is possible that a
553 regulatory linkage might exist between the energy state
554 of the cell and the need for alteration in protein
555 synthesis of the faster, α MyHC isoform. The overall
556 effect of a reduction in synthesis of the α MyHC would
557 be to reduce the rate of ATP consumption compatible
558 with a more anaerobic metabolism.

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