Creatine kinase B is necessary to limit myoblast fusion during myogenesis

Adriana Simionescu-Bankston,1,2*, Christophe Pichavant,2* James P. Canner,2 Luciano H. Apponi,2 Yanru Wang,2 Craig Steeds,2 John T. Olthoff,3 Joseph J. Belanto,3 James M. Ervasti,3 and Grace K. Pavlath2
1Graduate Program in Biochemistry, Cell and Developmental Biology, Emory University School of Medicine, Atlanta, Georgia; 2Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia; and 3Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota

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A variety of extracellular, cell surface, and intracellular molecules act to finely coordinate the cellular and molecular events that influence the ability of mammalian myoblasts to fuse (1, 65). During myotube formation, some cell surface (ADAM12, integrins, kindlin-2, cadherins), membrane (cholesteryl, phosphatidylinerine), and intracellular (actin, β-catenin, diacylglycerol kinase, EB3, nonmuscle myosin 2A, myoferlin, Rac1, syntrophin) molecules become spatially restricted to specific cellular domains (2, 8, 13, 16–18, 37, 44, 47, 62, 73, 76, 77). A number of these molecules are localized to cell-cell contact sites in opposing muscle cells, whereas other molecules localize to cell-cell contact sites between two muscle cells but in only one of the cells (50). Although the exact reasons for such localization during myogenesis are unknown, understanding why some molecules localize in this manner may provide valuable insights into the process of myoblast fusion.

We previously reported that creatine kinase B (CKB), the brain isoform of cytosolic CK enzymes, is prominently localized to myotube ends in vitro (49). CKB catalyzes the transfer of a phosphate group from phosphocreatine to ADP, thereby replenishing local cellular ATP pools at sites of high ATP turnover (81). These findings suggested that the ends of myotubes are sites of high ATP demand. However, the molecules that require ATP generated by CKB for their cellular function are unknown. Such molecules may be functionally important for myogenesis. Interestingly, CKB can also localize to specific cellular regions in nonmuscle cells. CKB transiently accumulates in membrane ruffles of astrocytes and fibroblasts during cell spreading and migration, and ablation of CKB activity negatively affects these two processes (36). In addition, CKB is transiently recruited to the phagocytic cup of macrophages during phagocytosis, where inhibition of CKB activity diminishes actin accumulation (35). Finally, CKB localizes to inner ear hair cells, and CKB-deficient mice exhibit hearing loss (63). Together, these studies indicate CKB activity is required at specific cellular locations in some cell types for various functions. Further examination of CKB function and localization specifically in muscle cells could enhance our understanding of the mechanisms underlying myotube formation.

To gain insights into the function of CKB at myotube ends, we first identified CKB-interacting proteins using a yeast two-hybrid screen. Subsequently, in-depth studies of two of these CKB-interacting proteins, α-skeletal actin and α-cardiac actin, predominant muscle actin isoforms, demonstrated their biochemical interaction and partial colocalization with CKB near the ends of myotubes in vitro. In contrast to other cell types, specific knockdown of CKB did not grossly affect actin polymerization in myotubes, suggesting other muscle-specific roles for CKB. Interestingly, knockdown of CKB resulted in significantly increased myoblast fusion and myotube size in vitro, whereas knockdown of creatine kinase M had no effect on these myogenic parameters. Our results suggest that localized CKB plays a key role in myotube formation by limiting myoblast fusion during myogenesis.

To gain insights into the function of CKB at myotube ends, we first identified CKB-interacting proteins using a yeast two-hybrid screen. Subsequently, in-depth studies of two of these CKB-interacting proteins, α-skeletal actin and α-cardiac actin, predominant muscle actin isoforms, demonstrated their biochemical interaction and partial colocalization with CKB near the ends of mouse myotubes in vitro. Finally, we used small-interfering RNA (siRNA) to decrease the levels of CKB and observed a significant increase in myoblast fusion and myotube size in cultured primary mouse muscle cells. Our...
results suggest CKB plays a key role in myotube formation by limiting myoblast fusion during myogenesis.

**Glossary**

Acta1 **α-Actin-1**  
ADAM12 A disintegrin and metalloprotease 12  
Arhgap23 Rho GTPase-activating protein 23  
Bin3 Bridging integrator 3  
CKB Creatine kinase, brain  
CKM Creatine kinase, muscle  
DM Differentiation media  
EB3 End-binding protein 3  
eMyHC Embryonic myosin heavy chain  
F-actin Filamentous actin  
FKHR Forkhead in human rhabdomyosarcoma  
G-actin Globular actin  
Gnb2 Gametogenetin-binding protein 2  
Hsp3 Heat shock protein 27-kDa protein 3  
Mbnl Muscleblind-like 3 (Drosophila)  
Rac1 Ras-related C3 botulinum toxin substrate 1  
Sept8 Septin-8  
Tmed10 Transmembrane emp24-like trafficking protein 10 (yeast)

**MATERIALS AND METHODS**

**Primary muscle cell culture.** Myoblasts were isolated from the hind limb muscles of C57BL/6J-129/SvJ, C57BL/6, or BALB/c mice as previously described with the exception of a Percoll gradient (7). Cultures were >95% myogenic as defined by MyoD immunostaining. Cells were cultured in growth media and switched to differentiation media (DM) for 24 or 40–48 h as previously described (66). Experiments involving animals were performed in accordance with approved guidelines and ethical approval from Emory University’s Institutional Animal Care and Use Committee.

**Immunoblotting for CKB and creatine kinase M.** Proteins were extracted from primary mouse muscle cells at different time points using RIPA-2 buffer, and samples were resolved by SDS-PAGE and immunoblotted. Primary antibodies against CKB/creatine kinase M (CKM) (gift from Dr. Theo Wallimann, Institute of Cell Biology, Swiss Federal Institute of Technology Zurich) or heat shock protein (HSP)-90 (Santa Cruz Biotechnology) were detected using the appropriate horseradish peroxidase (HRP)-conjugated IgG (Jackson Immunoresearch) secondary antibodies as previously described (19).

**Plasmid construction.** To generate an NH2-terminal LexA-CKB bait construct for yeast two-hybrid assays, a truncated cDNA fragment encoding the human CKB gene (accession no. NM_001823.4) (9) was generated using PCR and cloned into the pBTM116 vector (4).

To generate an NH2-terminal green fluorescent protein (GFP)-tagged CKB construct for coimmunoprecipitation experiments, a cDNA fragment encoding the full-length human CKB gene was generated using PCR and cloned into the pEGFP-C2 vector (Clontech). To generate NH2-terminal Myc-tagged constructs of the mouse genes of interest for coimmunoprecipitation experiments, cDNA fragments encoding the full-length mouse genes together with Myc were generated using PCR and cloned into a pcDNA3 plasmid (Clontech), or directly cloned into an NH2-terminal Myc tag-containing pcDNA3 plasmid.

To generate an NH2-terminal FLAG-copGFP-tagged CKB construct for actin-binding assays, a cDNA fragment encoding the full-length human CKB gene together with copGFP and FLAG was cloned into pENTR/D-TOPO (Invitrogen), recombined into pDEST8 (Invitrogen), and expressed in Sf9 insect cells using the Bac-to-Bac system (Invitrogen). To generate an NH2-terminal FLAG-tagged Fascin-1 construct, a cDNA fragment encoding the mouse full-length Fscn1 together with FLAG was cloned into pENTR/D-TOPO (Invitrogen), recombined into pDEST8, and expressed in S9 cells as previously described (54). For all experiments, fusion protein expression was verified by immunoblotting.

**Yeast two-hybrid assays.** The yeast two-hybrid screen was performed using Saccharomyces cerevisiae strain L40 [MATa his3 trp1 leu2 ade2 lys2::(lexA op)3-HIS3 ura3::(lexA op)3-lacZ] that harbors the HIS3 and lacZ reporter genes (27). The strain was initially transformed with the LexA-CKB bait construct in the pBTM116 plasmid (4), containing the TRP1 marker. Once expression of the bait protein in yeast was verified using immunoblotting with an anti-LexA antibody (Santa Cruz), L40 cells containing LexA-CKB were transformed with a Matchmaker 17-day-old mouse embryo cDNA library (Clontech) fused to the GAL4 activation domain in the pACT2 prey plasmid (Clontech), containing the LEU2 marker. Subsequently, transformants were plated onto selective medium (lacking Leu, Trp, and His) and incubated for 5 days at 30°C. His+ transformants were further screened for the formation of blue colonies in the β-galactosidase filter assay with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). The library plasmids from positive colonies were isolated, electrophoresed into Escherichia coli HB101 cells, and retransformed into L40 cells containing the LexA-CKB construct, and blue colony formation assays were then repeated for plasmid linkage. Plasmid DNA isolated from positive clones was sequenced to identify the genes encoding the interacting proteins.

**RT-PCR.** Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions, followed by treatment with DNase I (Life Technologies). Subsequently, total RNA (2.5 μg) was reverse transcribed, and PCR was performed as previously described (48), with the exception of using Tag DNA polymerase (Qiagen) and the primers listed in Table 1. To control for genomic contamination, all primers spanned an intron-exon boundary for the genes that contained one. As a control, 18S cDNA was extracted from primary mouse muscle cells at different time points by treatment with DNase I (Life Technologies). Subsequently, total RNA was isolated and visualized as previously described (48). PCR reactions were performed on a minimum of two independent isolates.

**Immunoprecipitation.** For coimmunoprecipitation experiments, HEK 293 cells were cotransfected with 10 μg pE6GFP-CBK/C2 plasmid and 10 μg pCDNA3 plasmid containing the Myc-tagged gene of interest using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. At 48 h posttransfection, cells were harvested in RIPA-2 buffer containing protease inhibitors. Cell lysates were then centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was subjected to immunoprecipitation.

**Table 1. RT-PCR primers used to study mRNA expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer Sequence</th>
</tr>
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</table>
| Acta1 | NM_001272041.1 | Fwd: ATGGTGCAAGAAAGAGAGACC  
Rev: CGGAATTTCTGAGAATTCTGGGCTG*  
| Aghaq23 | NM_021493.2 | Fwd: AGGGAGAAGAATTCCTTGAGAT  
Rev: GAGGGAGAAATTCCTGGGCTG  
| Bin3 | NM_021238.3 | Fwd: GGGGATCTGAAATCTGGAG  
Rev: GTGGAGCGTGGTGAATATGCG  
| Gngbp2 | NM_153144.2 | Fwd: GTGTAGGAAATTCTGGAGAACA  
Rev: TTTGTGCACTGACGAGCAT  
| Hsp3 | NM_019960.2 | Fwd: AGTGGCTTACACAGAGAGAAGT  
Rev: GGGTTTGGCTATCTGGCTTGC  
| Mbnl | NM_134163.4 | Fwd: TTAACTGTCAGTGCCTTTCC  
Rev: CGGGCTTATGTTGACATTCAT  
| Sept8 | NM_033144.2 | Fwd: TCTTCAGACGAGACCTTTGGAG  
Rev: GCTGGATAGAATGAGAGGCAA  
| Tmed10 | NM_026775.4 | Fwd: ACAAGTGTGCTGGGCGCATAT  
Rev: TTAACAAATGAGGTCTGGGAAAG  |

Fwd, forward; Rev, reverse. *Primer contains the EcoRI cloning site (bold) and extra nucleotides (italics).
To immunoprecipitate GFP-CKB, Dynabeads Protein-A Kit (Invitrogen) was used according to the manufacturer’s instructions. Briefly, rabbit anti-FLAG antibody (Invitrogen) or control rabbit IgG antibody (Santa Cruz) was cross-linked to the beads before the addition of protein extract (300–400 μg). After the beads were washed, the immunoprecipitate was eluted, followed by SDS-PAGE and immunoblotting.

To immunoprecipitate Myc-tagged proteins, Protein A/G PLUS-Agarose beads (Santa Cruz) were used according to the manufacturer’s instructions. Briefly, protein extract (500 μg) was incubated with mouse anti-Myc antibody (Santa Cruz) or control mouse IgG antibody (Santa Cruz), and then protein A/G PLUS-Agarose bead slurry was added. After the beads were washed, the supernatants were resuspended in SDS loading buffer and subjected to SDS-PAGE and immunoblotting.

Samples were resolved by SDS-PAGE, and immunoblots were performed as described (20), using a nitrocellulose membrane (Bio-Rad Laboratories). Primary antibodies against GFP (Invitrogen) or Myc (Covance) were detected using the appropriate HRP-conjugated IgG (Jackson ImmunoResearch) secondary antibodies.

Protein expression and purification for actin-binding assays. Recombinant baculovirus was transfected into small cultures of SF9 insect cells using CellFectin II (Invitrogen). Four days later, recombinant baculovirus was harvested, and the transfected cells were analyzed for FLAG-copGFP-CKB or FLAG-Fascin-1 protein expression by immunoblotting using the M2 anti-FLAG antibody (Sigma). After verification of fusion protein expression, large cultures (250 ml) were incubated for 3 days with amplified baculovirus before being harvested for protein purification. Cells were lysed and protein purified using anti-FLAG M2 affinity beads (Sigma) as previously described (60). Purified protein was dialyzed against PBS, concentrated, and used for in vitro actin-binding assays.

F-actin-binding assay. Purified α-skeletal actin (Cytoskeleton) was used for in vitro cosedimentation assays as previously described (26). Briefly, 0.5 μM FLAG-copGFP-CKB or 0.5 μM FLAG-Fascin-1 was incubated with 0–15 μM F-actin and subjected to high-speed centrifugation. Equal volumes of the supernatant (S) and the pellet (P) F-actin filaments together with the interacting protein were resolved by SDS-PAGE, stained using Coomassie blue, and imaged with the Odyssey infrared scanner (Li-cor). The relative amount of F-actin-bound protein was determined by densitometric analysis, and quantified as the P/(S + P) ratio. Data were plotted and fitted using nonlinear regression analysis (Graph-Pad Prism). Two independent experiments were performed with different FLAG-tagged protein and F-actin preparations.

G-actin-binding assay. Purified nonfilamentous G-actin (1 μM; Cytoskeleton) was incubated in the presence or absence of 1 μM purified FLAG-copGFP-CKB or anti-FLAG M2 affinity beads (Sigma) for 1 h at 4°C in G buffer (5 mM Tris-HCl pH 8.0, 0.2 mM CaCl₂, 0.2 mM ATP, 0.5 mM DTT). The beads were then pelleted, and the supernatant was collected. Subsequently, the beads were washed, and FLAG-copGFP-CKB was eluted with 0.1 mg/ml FLAG peptide (University of Minnesota Genomics Center). Equal volumes of starting material, FLAG-depleted supernatant, and FLAG elution were electrophoresed using SDS-PAGE and stained and imaged as described above.

Immunostaining. To detect CKB, myoblasts were differentiated for 0, 18, or 40 h in DM, fixed, and immunostained as previously described (49), using the tyramide signal amplification reagent (Tyramide Signal Amplification kit; Perkin Elmer). To detect CKB, myoblasts were differentiated for 0, 18, and 48 h in DM, fixed, and immunostained with rabbit anti-CKM (a gift from Dr. Theo Wallimann) and Alexa 594-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

For CKB and α-sarcomeric actin colocalization studies, myoblasts were differentiated for 40 h in DM and immunostained for CKB as previously described (49). Immunostaining for α-sarcomeric actin was performed using anti-α-sarcomeric actin (Sigma) antibody, and Alexa 594-conjugated donkey anti-mouse IgG (Jackson Immunoresearch). Nuclei were counterstained with DAPI.

siRNA transfection and myogenesis assays. Primary muscle mouse cells were plated at 1.5 × 10⁶ cells/well on collagen-coated six-well plates. Control siRNA (Stealth siRNA negative control medium GC duplex; Invitrogen), CKB, or CKM siRNAs (Invitrogen) were transfected at 120 nM using Lipofectamine 2000 (Invitrogen) for 5 h on two subsequent days. Two days after the last transfection, cells were trypsinized and plated at 2 × 10⁶ cells/well on 40 μg/ml entactin-collagen IV-laminin (ECL; Milipore) coated six-well plates and switched to DM for 24 or 48 h. In all subsequent assays, three to five independent isolates were analyzed. CKB siRNA no. 1: 5'-UGGAC-UUGAGAGAGUUUGCUG-3', CKB siRNA no. 2: 5'-UUG-GAUGCAGUGGAGAUGUAC-3', CKM siRNA: 5'-AUUCUCCUCAAUUCUGCGACCCA-3'. To determine the differentiation index, the fusion index, and the cell surface area, muscle cells were fixed and immunostained with an antibody against embryonic myosin heavy chain (eMyHC) as previously described (28).

Migration of transfected primary mouse muscle cells was quantified using time-lapse microscopy as previously described (30). Briefly, cells were seeded at 9 × 10⁴ cells/collagen-coated 35-mm dish and switched to DM for ~21 h before imaging. Images were recorded using a camera (Qimaging) and OpenLab 5.5.2 (Improvement) software every 5 min for 3 h. Cell velocities were calculated in micrometers per hour using ImageJ 1.46 [National Institutes of Health (NIH)] software by tracking the paths of mononucleated cells. Twenty mononucleated cells were tracked for each sample in three independent experiments.

Experimental determination of F/G-actin ratio. To determine the effect of CKB inhibition on actin polymerization in muscle cells, quantification of F- and G-actin in cellular fractions was used (G-Actin/F-actin In Vivo Assay Biochem Kit; Cytoskeleton). Briefly, muscle cells were transfected with control siRNA or CKB siRNA as described above and harvested after 18 h in DM. Lysates were cleared by centrifugation at 500 g for 5 min at room temperature. Subsequently, supernatants were centrifuged at 100,000 g for 1 h at 37°C, which resulted in F-actin in the pellet and G-actin in the supernatant. Pellets were then resuspended in F-actin depolymerization buffer. Samples were resolved by SDS-PAGE, and actin was subsequently detected using immunoblotting. The F/G-actin ratio was calculated by measuring the actin band intensity in the pellet and supernatant using Image J (NIH).

Image acquisition. For analyzing differentiation and fusion assays, cell surface area, cell velocity, images were obtained using an Axiovert 200M microscope (Carl Zeiss MicroImaging) with a 0.3 numeric aperture (NA) ×10 Plan-Neofluar objective (Carl Zeiss MicroImaging) and were recorded using a camera (Qimaging) and OpenLab 5.5.2 (Improvement) software. For all other experiments, images were obtained using an Axioplan microscope (Carl Zeiss MicroImaging) with a 0.8 NA ×25 Plan-Neofluar objective (Carl Zeiss MicroImaging) and were recorded with a camera (Carl Zeiss MicroImaging) and Scion Image 1.63 software (Scion). The total number of nuclei per field was counted to ensure similar cell density. A total of 94–171 nuclei were analyzed per condition.

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analysis to determine significance between two groups was performed
using a Student’s t-test. Differences were considered to be statistically
significant at $P < 0.05$.

RESULTS

**CKB is localized in both myocytes and myotubes.** To study
CKB localization during in vitro myogenesis (Fig. 1A), we
performed immunostaining in primary mouse muscle cells. We
previously showed CKB is localized in both nascent and
mature myotubes (49), suggesting CKB activity is likely crit-
ical at the ends of myotubes. However, we show here that CKB
also localized to the ends of elongated myocytes (Fig. 1B),
suggesting that CKB activity is also important in myocytes
before fusion. Thus, CKB may provide ATP for various mol-
ecules at the ends of differentiated muscle cells throughout
myotube formation. In contrast, CKM, the muscle isoform of
cytosolic CK enzymes, which localizes to the contractile ap-
paratus (61) and exhibits functional redundancy with CKB in
replenishing ATP (58), was absent from the ends of differen-
tiated muscle cells (Fig. 1C). Further differences between CKB
and CKM were noted by immunoblot, since CKB was ex-
pressed at higher levels than CKM at both 24 and 48 h of
differentiation (Fig. 2). The reciprocal localization of CKB and
CKM, together with their differential expression in muscle
cells, suggests distinctive functions for these two enzymes
during myotube formation. Given the localization of CKB to
the ends of differentiated muscle cells, a proposed site of

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Fig. 1. Creatine kinase B (CKB) and M (CKM) local-
azation during in vitro myogenesis. A: during in vitro
myogenesis, myoblasts differentiate into myocytes,
which fuse to one another to form nascent myotubes.
Subsequently, myocytes fuse with nascent myotubes,
giving rise to mature myotubes. B and C: pure cultures
of mouse muscle cells were immunostained for CKB or
CKM at various stages of myogenesis. CKB localized
throughout the cytoplasm in myoblasts, but near the
ends of myocytes and myotubes (arrowheads). In con-
trast, CKM was not expressed in myoblasts, and was
absent from the ends of both myocytes and myotubes (arrowheads). Nuclei were counterstained with 4’,6-
diamidino-2-phenylindole (DAPI). Bar, 50 μm.

Fig. 2. CKB and CKM protein expression during myogenesis. Primary mouse
muscle cells were analyzed by immunoblot for CKB and CKM at the indicated
time points. Tubulin was used as loading control. Arrowheads indicate the
CKB and CKM bands. CKB was the predominant cytosolic CK isoform at both
time points of differentiation. Both samples were derived and processed at the
same time and immunoblotted on the same membrane.
fusion in vitro (52), we focused our subsequent studies on CKB.

Screen for CKB-interacting proteins by yeast two-hybrid assay. To gain further insights into the function of CKB at the ends of differentiated muscle cells, we performed a yeast two-hybrid screen using the LexA-CKB fusion protein as bait to identify novel CKB-interacting proteins. Because we were unable to express the full-length CKB protein in yeast, we used a previously described (9) NH2-terminally truncated CKB construct (Fig. 3A) that includes the isoform-specific box 260 that distinguishes CKB from CKM (71), and the highly conserved cysteine-283 near the catalytic site. We confirmed expression of LexA-CKB in yeast by immunoblotting (Fig. 3B). Using this bait, we screened $1.2 \times 10^6$ clones from an embryonic day 17 mouse cDNA library for CKB-interacting proteins. Eight positive clones were isolated based on their ability to grow in SC-Histidine media and confirmed by activation of the lacZ reporter (Fig. 3C). No autoactivation of the lacZ reporter was observed for these clones (data not shown).

The positive clones obtained in the yeast two-hybrid screen (Fig. 3C) encode proteins with a diversity of functions, including regulation of the actin cytoskeleton (Table 2). These results are significant given the importance of actin regulation for several steps in myotube formation, including elongation, migration, and fusion (1). We identified α-skeletal actin, a skeletal muscle-specific actin isoform (53), as a putative CKB-interacting protein. Additional putative CKB-interacting proteins with roles in actin dynamics include bridging integrator 3 (Bin3), heat shock protein 27 kDa protein 3 (Hsp3), septin-8, and Rho GTPase-activating protein 23 (Arhgap23). N-BAR domain proteins, such as Bin3, link membrane dynamics to the actin cytoskeleton (21, 57). In addition, we showed that Bin3 is important for lamellipodia formation and muscle cell motility, as well as myotube formation and growth (66). Small heat shock proteins, such as Hsp3, inhibit actin polymerization by acting as capping proteins or may protect the actin cytoskeleton against disruption induced by various stressful conditions (42). Septin family proteins, such as septin-8, are small GTP-binding proteins that can self-assemble into filaments and rings (41), help organize actin bundles (33), and coordinate changes in cytoskeletal and membrane organization (80). RhoGAP proteins, such as Arhgap23, negatively regulate the function of RhoGTPases (40) with critical roles in modulating actin dynamics (24). Finally, several proteins identified in our yeast two-hybrid screen are involved in regulating cellular processes other than the actin cytoskeleton. Transmembrane emp24-like trafficking protein 10 (Tmed10) and gametogenetin-binding protein 2 (Ggnbp2) are involved in intracellular protein trafficking (72, 83), whereas muscleblind-like RNA-binding proteins, such as muscleblind-like 3 (Mbnl3), inhibit muscle differentiation and regulate alternative splicing of pre-mRNA (25, 38, 69, 74). Overall, the proteins identified in our screen

Table 2. CKB-interacting proteins identified in the yeast two-hybrid screen

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Protein Name</th>
<th>Type of Molecule</th>
<th>Function*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acta1</td>
<td>α-Skeletal-actin (14)</td>
<td>Skeletal muscle-specific cytoskeletal protein</td>
<td>Sarcomere component</td>
</tr>
<tr>
<td>Bin3</td>
<td>Bridging integrator 3 (56, 67)</td>
<td>BAR domain protein</td>
<td>Actin polymerization</td>
</tr>
<tr>
<td>Hsp3</td>
<td>Heat shock 27 kDa protein 3 (42)</td>
<td>Small heat shock protein</td>
<td>Actin polymerization</td>
</tr>
<tr>
<td>Sept8</td>
<td>Septin-8 (29, 33, 34)</td>
<td>GTP-binding protein</td>
<td>Actin polymerization</td>
</tr>
<tr>
<td>Arhgap23</td>
<td>Rho GTPase-activating protein 23 (22, 51, 55, 84)</td>
<td>GTPase-activating protein towards Rho/Rac/Cdc42</td>
<td>Intracellular protein trafficking</td>
</tr>
<tr>
<td>Tmed10</td>
<td>Transmembrane emp24-like trafficking protein 10 (yeast) (72)</td>
<td>Type I membrane protein</td>
<td></td>
</tr>
<tr>
<td>Ggnbp2</td>
<td>Gametogenetin-binding protein 2 (83)</td>
<td>Zinc finger protein</td>
<td>Intracellular protein trafficking</td>
</tr>
<tr>
<td>Mbnl3</td>
<td>Muscleblind-like 3 (Drosophila) (25, 38, 69, 74)</td>
<td>RNA-binding protein</td>
<td>Alternative splicing</td>
</tr>
</tbody>
</table>

*Because many of these proteins have several functions, only the function most relevant for myogenesis is listed. In addition, some of these protein functions (referenced in the table) are only predicted, or have only been shown for some family members.
regulate a number of highly ATP-dependent processes, which could require a localized source of ATP provided by CKB in muscle cells.

Because our goal was to gain insights into CKB function in muscle cells, we next examined whether the candidate proteins identified in our yeast two-hybrid screen using a nonmuscle library were expressed in muscle cells. We analyzed the expression of these candidates in primary mouse myoblasts differentiated into nascent myotubes (24 h) or mature myotubes (48 h) by RT-PCR. The genes identified in our screen were expressed at all stages of myogenesis, except 18S rRNA coding for \( \text{Acta1} \), which was present exclusively in differentiated muscle cells (Fig. 4).

CKB interacts with \( \alpha \)-skeletal actin and actin regulatory proteins. Given the importance of actin regulation for myoblast fusion (23, 32, 47, 77), we subsequently focused on studying the interaction of CKB with \( \alpha \)-skeletal actin, which was present exclusively in differentiated muscle cells (Fig. 4).

To determine whether the interaction between CKB and \( \alpha \)-skeletal actin is direct, we performed in vitro binding assays with purified proteins. Because actin can exist in either a filamentous (F-actin) or globular (G-actin) state, we tested the interaction of CKB with both forms of \( \alpha \)-skeletal actin. Incubation of a fixed amount of purified FLAG-copGFP-CKB with increasing amounts of F-actin, followed by high-speed centrifugation, indicated CKB did not measurably cosediment with F-actin (Fig. 6, A and B), whereas the positive control FLAG-

\[ \text{Fig. 4. Candidates isolated in the yeast two-hybrid screen are expressed in muscle cells. mRNAs for the genes identified in the yeast two-hybrid screen were present in primary mouse muscle cells using RT-PCR. All mRNAs except for Acta1 were expressed throughout differentiation, whereas Acta1 was only expressed at 24 and 48 h in differentiation media (DM). 18S rRNA was used as an internal control; } n = 2–3 \text{ independent isolates.} \]

\[ \text{Fig. 5. CKB interacts with } \alpha \text{-skeletal actin but not with } \beta \text{-cyto-actin or } \gamma \text{-cyto-actin isoforms. Coimmunoprecipitation experiments were performed using green fluorescent protein (GFP)-CKB and various Myc-tagged actin fusion proteins. GFP-CKB coimmunoprecipitated with Myc-} \alpha \text{-skeletal actin (A) and Myc-} \alpha \text{-cardiac actin (B) but not with Myc-} \beta \text{-cyto-actin (C) or Myc-} \gamma \text{-cyto-actin (D) as shown by immunoblot (IB). Immunoprecipitations (IP) were performed using either GFP or Myc antibodies. As a negative control, IgG was substituted for the primary antibody. Samples for each actin isoform were derived and processed at the same time and immunoblotted on the same membrane.} \]
Fascin-1 protein did (Fig. 6B). Similarly, purified FLAG-copGFP-CKB did not coimmunoprecipitate with purified G-actin using an anti-FLAG antibody (Fig. 6C). These results indicate that CKB likely interacts with α-skeletal actin indirectly via intermediary proteins.

Because α-skeletal actin is only one of six mammalian actin isoforms, which include the other muscle isoforms (α-cardiac actin, α-smooth-actin, γ-smooth-actin) and the cytoplasmic isoforms (β-cyto-actin, γ-cyto-actin) (53), we next determined whether CKB displays specificity in binding to any of these actin isoforms by coimmunoprecipitation. We focused on the isoforms expressed to a significant degree in cultured skeletal muscle cells during myogenesis, namely α-cardiac actin, β-cyto-actin, and γ-cyto-actin. α-Cardiac actin mRNA is rapidly induced upon differentiation and decreases as myotube maturation proceeds (3); in contrast, β-cyto-actin and γ-cyto-actin mRNA levels decline rapidly when differentiation begins (3). To determine whether CKB interacted with these actin isoforms biochemically, we expressed full-length GFP-CKB and Myc-tagged-actin constructs in HEK 293 cells and performed coimmunoprecipitation experiments. Immunoprecipitation using either GFP or Myc antibodies, followed by immunoblotting, indicated that GFP-CKB interacted with Myc-α-cardiac actin (Fig. 5B), but not with Myc-β-cyto-actin (Fig. 5C) or Myc-γ-cyto-actin (Fig. 5D). Together, these data demonstrate that CKB interacts with α-actin isoforms, but not with β- or γ-actin isoforms, in mammalian cells.

Finally, we also performed coimmunoprecipitation experiments to confirm the interaction between CKB and actin-regulatory proteins identified in our yeast two-hybrid screen (Table 2). We found Myc-Bin3 (Fig. 7A), Myc-Septin-8 (Fig. 7B), and Myc-Hsp3 (Fig. 7C) also coimmunoprecipitated with GFP-CKB in HEK 293 cells. These data indicate that in mammalian cells CKB can interact with multiple molecules important for actin dynamics, a highly ATP-dependent process (39).

Decreased levels of CKB do not alter actin polymerization in myocytes. We next analyzed the functional relevance of CKB activity for the actin cytoskeleton in muscle cells. We initially examined the colocalization of CKB with α-skeletal actin and α-cardiac actin, the two predominant muscle actin isoforms (57). Primary mouse myoblasts were differentiated for 24 h into myotubes and costained for CKB and with an antibody that recognizes both α-skeletal actin and α-cardiac actin, which are 99% identical and together are commonly referred to as α-sarcomeric actin (75). Immunofluorescence analyses revealed that CKB and α-sarcomeric actin partially colocalized near myotube ends (Fig. 8). Given the role of CKB in replenishing local ATP levels, we subsequently hypothesized that decreasing the levels of CKB would significantly impact actin polymerization given that actin filament assembly is highly ATP dependent (39). To test this hypothesis, we employed an siRNA-based approach to decrease the levels of CKB by transfecting primary mouse muscle cells with either control or CKB siRNA. Immunoblot analyses indicated that CKB siRNA no. 1 reduced the levels of CKB by 77% but did not affect CKM protein levels (Fig. 9A). Subsequently, we quantified actin in the soluble (G) vs. insoluble (F) fractions of transfected primary mouse muscle cells after 18 h of differentiation into myocytes. Because most of the actin cytoskeleton in myotubes is resistant to drugs causing actin depolymerization (79), we hypothesized that any potential changes in actin polymerization due to CKB knockdown would be most observable in myocytes. However, no difference in the F/G-actin ratio was observed between control and CKB siRNA-treated muscle cells (Fig. 9, B and C). These results are in contrast to the decreased actin polymerization observed in nonmuscle cells.
upon inhibition or loss of CKB activity (12, 15, 35), suggesting other muscle-specific roles for CKB.

Knockdown of CKB, but not CKM, enhances myotube formation. To study the functional significance of CKB localization during myogenesis, primary mouse myoblasts were transfected with CKB siRNA no. 1 or control siRNA. In parallel, we also transfected myoblasts with CKM siRNA. Subsequently, parameters of myogenesis at various times of differentiation were analyzed. At 24 and 48 h of differentiation, cultures were immunostained for embryonic myosin heavy chain, a marker of myogenic differentiation. At both time points, myotubes appeared larger in cultures transfected with

Fig. 7. CKB interacts with actin regulatory proteins. GFP-CKB coimmunoprecipitated with Myc-Bin3 (A), Myc-Septin-8 (B), and Myc-Hsbp3 (C) as shown by immunoblot. IP were performed using either GFP or Myc antibodies. As a negative control, IgG was substituted for the primary antibody. Samples for each actin regulatory protein were derived and processed at the same time and immunoblotted on the same membrane.

Fig. 8. CKB and α-sarcomeric actin colocalize near the ends of myotubes. CKB did not extend all the way to the tips of myotubes (left, green), whereas α-sarcomeric actin did (middle, red). The merge shows colocalization of the two proteins near the ends of myotubes (right, arrowhead). Nuclei were counterstained with DAPI. Bar, 50 μm.

Fig. 9. Small-interfering RNA (siRNA)-mediated knockdown of CKB does not alter the F/G-actin ratio. A: in primary mouse muscle cells transfected with CKB siRNA (CKB no. 1) and differentiated for 24 h, CKB levels were decreased by 77 ± 5% compared with control (CTL) siRNA by immunoblot (lighter exposure). CKM levels were unchanged following CKB siRNA treatment (darker exposure). Heat shock protein (HSP)-90 was used as loading control. Arrowheads indicate the CKB and CKM bands. B: representative immunoblot showing actin in the soluble (G) vs. insoluble (F) fractions at 18 h of differentiation. C: quantification of the F/G-actin ratio from immunoblots. Data are means ± SE; n = 3 independent isolates with CTL and CKB siRNA.
CKB siRNA no. 1 compared with control siRNA (Fig. 10A), whereas cultures transfected with CKM siRNA (Fig. 10H) did not show differences in myotube size compared with control siRNA by embryonic myosin heavy chain immunostaining (data not shown). Quantitative analyses revealed that the fusion index (Fig. 10B) and the surface area of myotubes (Fig. 10C) were significantly increased at 24 and 48 h of differentiation, respectively, with CKB siRNA no. 1 but not with CKM siRNA (Fig. 10, I and J). Similar results were obtained with a different siRNA against CKB, CKB siRNA no. 2 (Fig. 11, A–D). These results indicate that myoblast fusion and myotube growth are enhanced with diminished levels of CKB, but not with diminished levels of CKM. Myotube formation is a multistep process in which early events such as differentiation, cell migration, and cell number can significantly impact myoblast fusion. Thus, we performed subsequent experiments to determine the effect of CKB knockdown on these parameters of myogenesis. CKB siRNA did not affect differentiation (Fig. 10D) or myocyte velocity during cell migration (Fig. 10E). A significant decrease in the number of nuclei at 24 h of differentiation was noted with CKB siRNA no. 1 (Fig. 10F) but not with CKB siRNA no. 2 (Fig. 11E). Decreased cell number would lead to decreased cell fusion, not the enhancement of cell fusion observed at this time point (Fig. 10B). No effect of either CKB siRNA on the number of nuclei was observed at 48 h of differentiation (Figs. 10G and 11F). Together, these results indicate that myoblast fusion and myotube growth are enhanced with diminished levels of CKB, but not with diminished levels of CKM.

**Fig. 10.** siRNA-mediated knockdown of CKB, but not CKM, enhances myogenesis. A: representative images of CTL siRNA and CKB siRNA-treated cells differentiated for 24 or 48 h and immunostained for embryonic myosin heavy chain (eMyHC). Arrowheads indicate network of myotubes suggestive of myotube-myotube fusion. Bar, 150 μm. B: at 24 h of differentiation, the fusion index was significantly increased with CKB siRNA. C: at 48 h of differentiation, the surface area of myotubes was significantly increased with CKB siRNA. AU, arbitrary units. D: differentiation was not altered with CKB knockdown. E: myocyte migration was not altered with CKB knockdown. The total number of nuclei was significantly decreased at 24 h (F) but not at 48 h (G) with CKB knockdown. H: in primary mouse muscle cells transfected with CKM siRNA and differentiated for 24 h, CKM levels were decreased by 63% on average compared with CTL siRNA by immunoblot. CKB levels were unchanged following CKM siRNA treatment. HSP-90 was used as loading control. Arrowheads indicate the CKB and CKM bands. I: at 24 h of differentiation, the fusion index was not significantly altered with CKM knockdown. J: at 48 h of differentiation, the surface area of myotubes was not significantly altered with CKM knockdown. Data are means ± SE; n = 3–5 independent isolates using CTL, CKB, and CKM siRNA with *P < 0.05.
HSP-90 was used as loading control.
no. 2 compared with control siRNA (CTL).
level of differentiation was reduced by
-60% in cells transfected with CKB siRNA
no. 2 compared with control siRNA (CTL).
HSP-90 was used as loading control. B:
representative images of CTL and CKB
siRNA no. 2-treated cells differentiated for
24 or 48 h and immunostained for eMyHC.
Bar, 150 μm. At 24 and 48 h of differen-
tiation, the fusion index (C) and the surface
area (D), respectively, were significantly in-
creased with CKB siRNA no. 2 compared
with CTL siRNA treatment. No difference in
the number of nuclei was noted at 24 or 48 h
of differentiation between CTL and CKB
siRNA no. 2-treated cells (E and F). Data are
means ± SE. n = 3–4 independent isolates
using CTL and CKB siRNA cells with *P <
0.05.

Fig. 11. Myogenesis is also enhanced using a
different siRNA against CKB. A: primary
mouse muscle cells were transfected with a
different CKB siRNA (CKB no. 2). By immu-
noblot, CKB levels were reduced by
-nuclear export (46). Inhibition of either prostacyclin or
ROCK1 in mouse muscle cells stimulates myoblast fusion (6,
11, 46), as we observed with knockdown of CKB.

Using a yeast two-hybrid screen, we identified a number of
novel CKB-interacting proteins, many of which are involved in
actin polymerization, an ATP-intensive process. We showed
two muscle-specific actin isoforms, α-skeletal actin and α-car-
diactin, interacted and partially colocalized with CKB near
myotube ends. However, knockdown of CKB did not result in
gross defects in actin polymerization as measured by immu-
noblots or by phalloidin staining (data not shown). In addition,
cell migration, a process dependent on actin remodeling, was
not altered in CKB siRNA-treated muscle cells, further sug-
gesting that actin polymerization is not disrupted at this stage
of myogenesis. In contrast, genetic or pharmacological inhibi-
tion of CKB results in changes of the actin cytoskeleton in
macrophages, osteoclasts, and cardiomyocytes (12, 15, 35).
Several factors could account for this difference between
skeletal muscle cells and other cell types. 1) Skeletal muscle
cells express a significant amount of CKM, whereas other cell
types generally do not. CKM activity may partially compensate
for the loss of CKB and prevent any changes in actin polymer-
ization in skeletal muscle cells. Indeed, siRNA-mediated
knockdown of both CKB and CKM in primary mouse skeletal
muscle cells abrogates the enhanced myotube size observed
with just CKB knockdown (49). 2) Small localized changes in
actin dynamics may occur at different stages of myogenesis
with CKB knockdown that were not observable in the assays
used here. A number of dynamic actin-based localized cellular
structures play important roles at various steps during myotube
formation, including fusion. For example, before fusion, mus-
cle cells exhibit lamellipodia, actin-based protrusions contain-
ning branched actin filaments, which are associated with motil-

DISCUSSION

The dramatic relocalization of CKB to the ends of differen-
tiated muscle cells during myotube formation suggested CKB
activity, which replenishes local ATP pools, is necessary for
the function of molecules that turn over ATP rapidly at these
sites. These molecules may consume ATP too rapidly for
replenishment by diffusion due to the large size of myotubes;
therefore, the ends of differentiated muscle cells may require
localized ATP production. As discussed below, our studies
provide insights into CKB function during myotube formation.

The major finding of this study is that CKB activity acts to
limit myoblast fusion during myogenesis, since knockdown of
CKB with siRNA resulted in significantly increased myoblast
fusion and myotube size. CKB siRNA treatment did not alter
muscle cell differentiation, migration, or viability, suggesting
that CKB activity may be important for ATP-intensive mole-
cules during the fusion process itself. Molecules that help limit
cell fusion during myogenesis may be critical to ensure an
orderly process of fusion and prevent a “traffic jam” of fusion-
competent cells. The web of interconnected myotubes ob-
served with CKB knockdown at later time points suggests that
CKB activity may also be important for limiting myotube-
myotube fusion. This action may help funnel differentiated
myocytes to fuse with myotubes, ensuring the growth of
individual myotubes. Such an action would be critical in vivo
to regenerate the proper cylindrical structure of myofibers after
injury. Only two molecules to date have been shown to nega-
tively regulate myoblast fusion. Prostacyclin, a secreted mem-
brane protein, helps to maintain myoblast prolif-
eration and prevent terminal commitment to differentiation
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tively regulate myoblast fusion. Prostacyclin, a secreted mem-
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eration and prevent terminal commitment to differentiation
(11, 46). ROCK1 activity results in phosphorylation of the
transcription factor forkhead in human rhabdomyosarcoma
(FKHR) required for myoblast fusion (5), leading to FKHR
nuclear export (46). Inhibition of either prostacyclin or
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cle cells exhibit lamellipodia, actin-based protrusions contain-
ing branched actin filaments, which are associated with motil-

ity (68). Later in myogenesis, myoblasts align in preparation for fusion (78). During early alignment of elongated myoblasts (78), a cortical actin wall extends the length of the plasma membrane of the two aligned cells, possibly providing a temporal barrier for fusion until the myoblasts are completely aligned and ready to fuse (18). Fusing myoblasts then exhibit filopodia, exploratory extensions from the plasma membrane containing parallel bundles of actin filaments (59), which are used to connect fusing myoblasts along the entire length of the membrane contact (70). Finally, during late myogenesis, myotube-myotube fusion occurs between the leading edge of lamellipodia of one myotube and the lateral plasma membrane of the other (43). Disruption of actin polymerization in one of these dynamic structures may have occurred upon knockdown of CKB. 3) CKB interacts with various proteins in skeletal muscle cells, some of which positively or negatively regulate actin remodeling. In this scenario, both positive and negative actin regulators would be affected by CKB knockdown, but no net outcome on actin polymerization would be observed. In summary, our results suggest that CKB activity has specific roles in myogenesis, and further work will be required to identify the molecules whose function is altered with knockdown of CKB.

Our coimmunoprecipitation studies showed that CKB interacts only with muscle-specific isoforms of actin, but not with the ubiquitously expressed β-cyto-actin or γ-cyto-actin isoforms, which is a novel finding. α-Skeletal actin and α-cardiac actin are 99% identical (53), whereas β-cyto-actin and γ-cyto-actin are 98% identical and 93% identical to the sarcomeric actin isoforms (31). Many actin-binding proteins, including ezrin (82), βCAP73 (64), and γ-plastin (45), interact preferentially with nonmuscle actin isoforms, such as β-cyto-actin, but not with α-actin, by cosedimentation assays. Thus, very small differences among actin isoforms may be sufficient to confer differential binding affinities to various actin-binding proteins. However, how the specificity of the interaction between actin-binding proteins and various actin isoforms occurs is unknown.

We observed that CKB localizes near myotube ends, where it partially colocalizes with sarcomeric actins. Interestingly, CKB also localizes to specific cellular regions in conjunction with nonsarcomeric actins in nonmuscle cells. In spreading astrocytes, a fraction of CKB and F-actin jointly accumulates in membrane ruffles, where actin-based structures are most dynamic (36). In macrophages and microglia not undergoing phagocytosis, CKB localizes to the cytoplasm; however, in cells undergoing phagocytosis, a portion of CKB is cytoplasmic and another is found at nascent phagosomes, suggesting a partial shift in localization (35). F-actin is also found at nascent phagosomes in an almost complete overlap with CKB (35). These studies indicate CKB is localized to specific cellular regions of actin remodeling in multiple nonmuscle cell types.

CKB could become localized in differentiated muscle cells in multiple ways. For example, CKB mRNA and protein synthesis may be localized to the ends of differentiated muscle cells. Alternatively, CKB may be synthesized throughout the cell, but actin regulatory proteins identified in our screen, such as Bin3, may be responsible for CKB localization in muscle cells. However, CKB localization in myotubes was not altered in muscle cells lacking Bin3 (data not shown). Furthermore, latrunculin treatment did not affect CKB localization in myocytes (data not shown), suggesting F-actin is not structurally required for maintaining CKB at the ends of differentiated muscle cells. In contrast, CKB localization in astrocytes was F-actin dependent, since treatment with cytochalasin D, an inhibitor of actin polymerization (10), reduced the accumulation of both F-actin and CKB around fibronectin beads (36). These results suggest CKB localization is likely controlled by distinct mechanisms in different cell types. Further studies are required to identify the mechanisms regulating CKB localization in differentiated muscle cells. Identifying these mechanisms will enable disrupting CKB localization and studying the consequences of this disruption for muscle cell fusion and growth.

In summary, our data suggest local ATP produced by CKB near the ends of differentiated muscle cells plays an important role in regulating myotube formation. Future studies should be directed towards elucidating how CKB and its interacting partners work together to regulate myotube formation. Such studies will provide valuable insights into the regulation of myoblast fusion, and could further allow the development of therapies to enhance muscle growth.

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