Supporting Information

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SI Materials and Methods

Preparation of MyBP-C. Sf9 insect cells were infected with high-titer recombinant baculoviruses encoding FLAG-tagged full-length or truncated mouse MyBP-C (1), incubated 72 h postinfection. Full-length fast-skeletal (fsMyBP-C), slow-skeletal (ssMyBP-C) and cardiac (cMyBP-C), and truncated cardiac C0–C1 (i.e., C0-pa-C1, where pa is the linker rich in proline and alanine content), C0–C4 (i.e., C0-pa-C1-m-C2-C3-C4, where m is the phosphorylation motif), C5–C10 (i.e., lacking C0–C4), and ΔC0–C1 (i.e., m-C2-C10, thus lacking C0–C1), as depicted in Fig. 3*A*. Expressed protein was purified by anti-FLAG M2 affinity chromatography as previously described for the purification of full-length FLAG-tagged mouse utrophin except that Triton X-100 was omitted (2–5). The concentration of purified proteins was measured with the Bradford protein assay using BSA as a standard.

Preparation and Labeling of Actin. Actin was prepared from rabbit skeletal muscle (4, 6) and suspended in 100 mM NaCl, 2 mM $MgCl_2$, 0.2 mM ATP, 1 mM DTT, and 10 mM Tris, pH 8.0. For phosphorescence experiments, actin was labeled at C374 with erythrosin-5′-iodoacetamide (ErIA; AnaSpec) (4). Labeled F-actin (ErIA-actin) was stabilized against depolymerization and denaturation by adding one molar equivalent of phalloidin. The extent of labeling, determined by measuring dye absorbance and protein concentration, was 0.87 ± 0.11 mol dye/mol actin. The concentration of labeled actin was measured with the Bradford protein assay using unmodified actin as a standard, as attached dyes had a negligible effect on this assay (5).

In Vitro Phosphorylation of MyBP-C. Purified MyBP-C was phosphorylated with the catalytic subunit of PKA) Sigma), using 0.01 units of PKA per μg of MyBP-C for 1 h at 25 °C (1). Phosphorylation status of MyBP-C was assessed by Pro-Q Diamond phosphoprotein staining followed by Sypro-Ruby protein staining (Invitrogen). Untreated MyBP-C is not fully unphosphorylated. For example, cMyBP-C is expressed with mainly one site phosphorylated (7) and mainly three sites phosphorylated following treatment with PKA (1).

Actin Binding Analysis. Binding of proteins to actin filaments was measured using high-speed cosedimentation (8). Varying concentrations of MyBP-C were added to 5 μM actin, incubated for 30 min at 20 °C, and then centrifuged at 100,000 × *g* for 20 min. The resulting pellets and supernatants were separated by SDS/PAGE and stained with Coomassie blue. The fractions of free and bound protein were quantified by densitometry. K_d and B_{max} of binding were obtained by fitting data by the hyperbolic binding function:

$$y = B_{max}*[P]/(K_d + [P]), \quad \textbf{[S1]}$$

where y = bound MyBP-C (mol/mol actin) and [P] = free MyBP-C (μM). Attached dyes had a negligible effect on this assay, so TPA results were compared with and interpreted in light of the extensive studies of MyBP-C actin-binding properties by Rybakova et al. (1), using the same purified proteins and experimental conditions.

Electron Microscopy. Samples of phosphorescent actin, prepared for spectroscopic experiments, were diluted to 0.02 mg/mL in MyBP-buffer; applied to glow-discharged, collodion-, and carbon-film-coated copper grids; stained by 1% uranyl acetate; and observed with a JEOL 100 CX electron microscope at an accelerating voltage of 80 kV. Photographs were taken at 5,000× magnification, and digital copies of negatives were saved for viewing (6).

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Table S1. Effect of saturating concentration of MyBP-C variants on the parameters of actin TPA decay

	ϕ_1		ϕ_2		r_1		r_2		r_∞	
MyBP-C variant	(–) PKA	(+) PKA	(–) PKA	(+) PKA	(–) PKA	(+) PKA	(–) PKA	(+) PKA	(–) PKA	**(+) PKA**
None	183 ± 5	168 ± 10	27 ± 1	23 ± 1†	0.037 ± 0.001	0.041 ± 0.001†	0.040 ± 0.001	0.037 ± 0.003	0.034 ± 0.003	0.036 ± 0.006
Fast skeletal	233 ± 13*	119 ± 7†	17 ± 5*	17 ± 3	0.012 ± 0.001*	0.029 ± 0.001†	0.032 ± 0.001*	0.018 ± 0.003†	0.066 ± 0.012*	0.058 ± 0.006
Slow skeletal	136 ± 11*	177 ± 11	24 ± 4	22 ± 5	0.016 ± 0.001*	0.037 ± 0.001†	0.021 ± 0.002*	0.030 ± 0.003†	0.077 ± 0.011*	0.036 ± 0.006†
Cardiac	211 ± 13*	276 ± 14†	26 ± 2	29 ± 1	0.019 ± 0.003*	0.037 ± 0.001†	0.020 ± 0.003*	0.039 ± 0.001†	0.080 ± 0.010*	0.047 ± 0.005†
C0–C1	163 ± 7*	158 ± 5	24 ± 1	28 ± 1†	0.032 ± 0.002*	0.033 ± 0.001	0.037 ± 0.002	0.037 ± 0.002	0.039 ± 0.009	0.040 ± 0.005
C0–C4	151 ± 14*	167 ± 11	22 ± 1*	22 ± 1	0.026 ± 0.004*	0.031 ± 0.002	0.041 ± 0.002	0.044 ± 0.002	0.054 ± 0.014	0.043 ± 0.004
ΔC0–C1	205 ± 22	190 ± 13	22 ± 1	25 ± 1	0.012 ± 0.002*	0.036 ± 0.002†	0.029 ± 0.002*	0.038 ± 0.001†	0.069 ± 0.005*	0.041 ± 0.008†
C5–C10	291 ± 23*	280 ± 14	27 ± 3	30 ± 1	0.017 ± 0.002*	0.021 ± 0.001	0.022 ± 0.002*	0.025 ± 0.001	0.074 ± 0.005*	0.072 ± 0.003

Values are mean ± SEM ($n > 5$).

*Significant change in absence of PKA treatment compared with actin alone ($P < 0.05$).

†Significant change due to PKA treatment ($P < 0.05$).