Myosin Binding Protein C Slow is a Novel Substrate for Protein Kinase C (PKC) and A (PKA) in Skeletal Muscle

SUPPLEMENT

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Supplemental Figure Legends

SFig 1: A-B: Phosphorylation events mediated by PKC were identified in the NH_2-terminal construct via mass spectrometry analysis using LTQ. MS/MS scans of peptides carrying phosphorylated Ser-83 (A) and Thr-84 (B) identified by Bioworks-SEQUEST with Xcorr scores of 2.827 and 2.94, respectively, and manually validated are shown. An immunoreactive band of ~150 kDa was detected in homogenates prepared from adult FDB muscle, using affinity-purified antibodies recognizing phosphorylated Ser-83 (inset in panel A) or Thr-84 (inset in panel B), confirming that native MyBP-C slow undergoes phosphorylation at those amino acids *in vivo*. C: Schematic of a hypothetical MyBP-C slow protein containing all three novel insertions, highlighted in red, generated from a compilation of published sequences (NCBI accession numbers, EDL21488, HQ848554, HQ848555). Highlighted light and dark regions denote Ig and FNIII domains, respectively, while underlined regions indicate the three recombinant proteins, NH_2-terminal construct, C7-domain construct and COOH-terminal construct. Residues identified by MS/MS to be phosphorylated are shown in bold. D: *In* vitro kinase assays of wild type and mutant NH_2-terminal constructs treated with PKA and PKC were separated by 1-D SDS-PAGE and stained with the ProQ Diamond phospho-specific and Sypro Ruby total protein dyes. Wild

type (GST-NH_2 terminal construct), but not mutant forms in which Ser 59 and Ser 62 (GST-NH_2 terminal construct S59A/S62A) or Ser 83 and Thr 84 (GST-NH_2 terminal construct S83A/T84A) were tandemly mutated to Ala, stain positive with ProQ Diamond dye, following treatment with PKA or PKC, respectively, confirming the phosphorylation events identified by mass spectrometry.

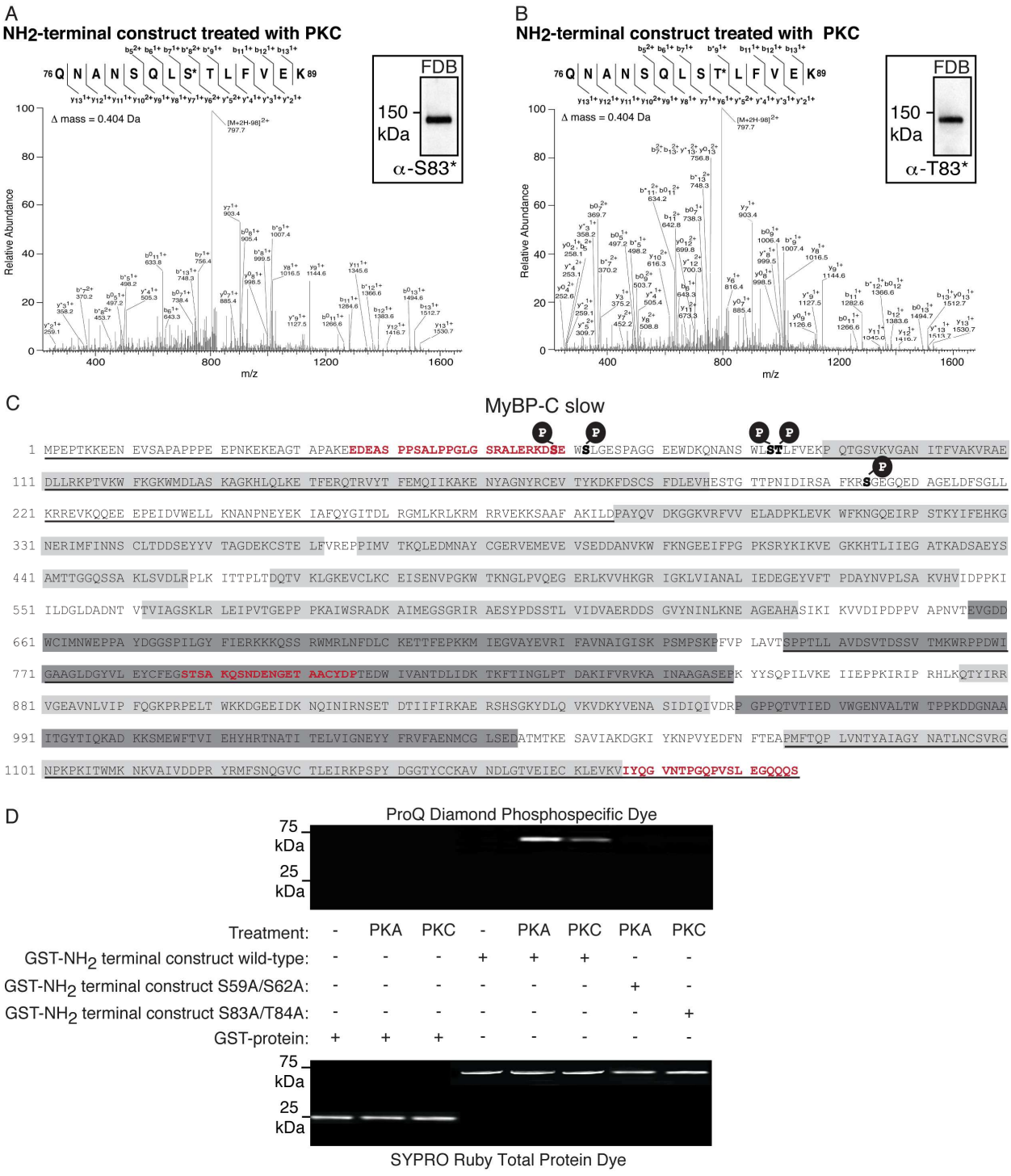
SFig 2: A-B: Portions of MyBP-C slow (A, top panel), fast (B, top panel) and GAPDH (A and B, bottom panels) were amplified using RT-PCR analysis and cDNA prepared from wild type and MDX FDB and TA muscles. Densitometry of the amplified fragments was used to calculate the relative amounts of MyBP-C slow and fast transcripts in normal and dystrophic muscles after being normalized to those of GAPDH; percent changes are reported in bar graphs (Students t-test, $p<0.01$). C-F: Localization of MyBP-C slow and fast in wild type and MDX skeletal muscle. Wild type (C1-C3 and E1-E3) and MDX (D1-D3 and F1-F3) TA muscles were co-stained with antibodies for MyBP-C slow (C1 and D1; green) or MyBP-C fast (E1 and F1; green) and the Rho-GEF domain of obscurin (C2 and D2, and E2 and F2; red), which specifically labels M-bands. The localization of MyBP-C fast is not altered in TA dystrophic muscle; however, MyBP-C slow is moderately affected in ~10% of myofibers.

Supplemental Table 1

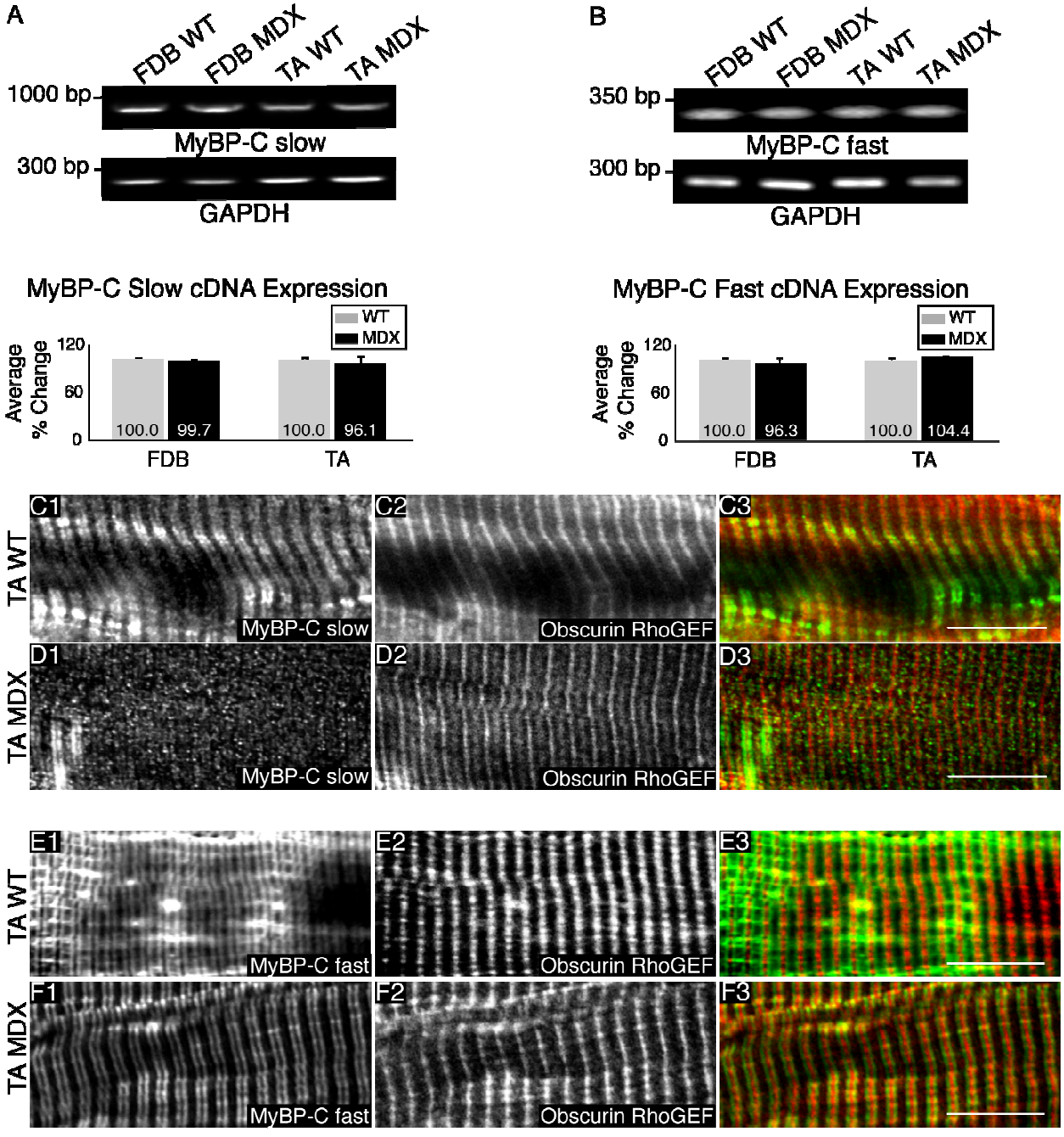
Directional Cloning Primers		
Amplicon	**Sense Primer**	**Antisense Primer**
NH_2-domain	ATGCCAGAACCCACTAAG	ATCGAGAATTTTTGCAAA
C7-domain	AGCCCTCCTACTCTTCTG	GGGCTCACTAGCCCCAGC
COOH-domain	CCCATGTTTACTCAACCT	CGACTGTTGCTGCCCCTC
MyBP-C fast	CCCAAGTTCCTGACA	TCACTGCGGCAC
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT

Mutagenesis Primers			
Mutation	**Template**	**Sense Primer**	**Antisense Primer**
NH_2-S59A domain	NH_2-domain	ATGGAGAGAAAAGATGCAGAATGGTCTCTTGGTGAG	CTCACCAAGAGACCATTCTGCATCTTTTCTCTCCAG
NH_2-S59A/S62A domain	NH_2-S59A domain	GAAAAAGATGCAGAATGGGCTCTTGGTGAGTCACCTGCTG	CAGCAGGTGACTCACCAAGAGCCCATTCTGCATCTTTTC
NH_2-S83A domain	NH_2-domain	GCCAACTCCCAGCTGGCCACCCTGTTTGTTGAAAAAC	GTTTTTCAACAAACAGGGTGGCCAGCTGGGAGTTGGC
NH_2-S83A/T84A domain	NH_2-S83A domain	CAACTCCCAGCTGGCCGCCCTGTTTGTTGAAAAACCTC	GAGGTTTTTCAACAAACAGGGCGGCCAGCTGGGAGTTG

SFig. 1


A
NH2-terminal construct treated with PKC
76Q N A N S Q L S* T L F V E K89
Δ mass = 0.404 Da
FDB
150 kDa
α-S83*
Relative Abundance
m/z
B
NH2-terminal construct treated with PKC
76Q N A N S Q L S T* L F V E K89
Δ mass = 0.404 Da
FDB
150 kDa
α-T83*
Relative Abundance
m/z
C
MyBP-C slow
1 MPEPTKKEEN EVSAPAPPPE EPNKEKEAGT APAKEEDEAS PPSALPPGLG SRALERKDSE WSLGESPAGG EEWDKQNANS WLSTLFVEKP QTGSVKVGAN ITFVAKVRAE
111 DLLRKPTVKW FKGKWMDLAS KAGKHLQLKE TFERQTRVYT FEMQIIKAKE NYAGNYRCEV TYKDKFDSCS FDLEVHESTG TTPNIDIRSA FKRSGEGQED AGELDFSGLL
221 KRREVKQQEE EPEIDVWELL KNANPNEYEK IAFQYGITDL RGMLKRLKRM RRVEKKSAAF AKILDPAYQV DKGGKVRFVV ELADPKLEVK WFKNGQEIRP STKYIFEHKG
331 NERIMFINNS CLTDDSEYYV TAGDEKCSTE LEVREPPIMV TKQLEDMNAY CGERVEMEVE VSEDDANVKW FKNGEEIFPG PKSRYKIKVE GKKHTLIIEG ATKADSAEYS
441 AMTTGGQSSA KLSVDLRPLK ITTPLTDQTV KLGKEVCLKC EISENVPGKW TKNGLPVQEG ERLKVVHKGR IGKLVIANAL IEDEGEYVFT PDAYNVPLSA KVHVIDPPKI
551 ILDGLDADNT VTVIAGSKLR LEIPVTGEPP PKAIWSRADK AIMEGSGRIR AESYPDSSTL VIDVAERDDS GVYNINLKNE AGEAHASIKI KVVDIPDPPV APNVTEVGDD
661 WCIMNWEPPA YDGGSPILGY FIERKKKQSS RWMRLNFDLC KETTFEPKKM IEGVAYEVRI FAVNAIGISK PSMPSKPFVP LAVTSPPTLL AVDSVTDSSV TMKWRPPDWI
771 GAAGLDGYVL EYCFEGSTSA KQSNDENGET AACYDPTEDW IVANTDLIDK TKFTINGLPT DAKIFVRVKA INAAGASEPK YYSQPILVKE IIEPPKIRIP RHLKQTYIRR
881 VGEAVNLVIP FQGKPRPELT WKKDGEEIDK NQINIRNSET DTIIFIRKAE RSHSGKYDLQ VKVDKYVENA SIDIQIVDRP GPPQTVTIED VWGENVALTW TPPKDDGNAA
991 ITGYTIQKAD KKSMEWFTVI EHYHRTNATI TELVIGNEYY FRVFAENMCG LSEDATMTKE SAVIAKDGKI YKNPVYEDFN FTEAPMFTQP LVNTYAIAGY NATLNCSVRG
1101 NPKPKITWMK NKVAIVDDPR YRMFSNQGVC TLEIRKPSPY DGGTYCCKAV NDLGTVEIEC KLEVKVIYQG VNTPGQPVSL EGQQQS
D
ProQ Diamond Phosphospecific Dye
75 kDa
25 kDa
Treatment: - PKA PKC - PKA PKC PKA PKC
GST-NH2 terminal construct wild-type: - - - + + + - -
GST-NH2 terminal construct S59A/S62A: - - - - - - + -
GST-NH2 terminal construct S83A/T84A: - - - - - - - +
GST-protein: + + + - - - - -
75 kDa
25 kDa
SYPRO Ruby Total Protein Dye

SFig. 2


A
FDB WT
FDB MDX
TA WT
TA MDX
1000 bp
MyBP-C slow
300 bp
GAPDH
B
FDB WT
FDB MDX
TA WT
TA MDX
350 bp
MyBP-C fast
300 bp
GAPDH
MyBP-C Slow cDNA Expression
WT
MDX
Average % Change
120
60
0
100.0
99.7
100.0
96.1
FDB
TA
MyBP-C Fast cDNA Expression
WT
MDX
Average % Change
120
60
0
100.0
96.3
100.0
104.4
FDB
TA
C1
C2
C3
TA WT
MyBP-C slow
Obscurin RhoGEF
D1
D2
D3
TA MDX
MyBP-C slow
Obscurin RhoGEF
E1
E2
E3
TA WT
MyBP-C fast
Obscurin RhoGEF
F1
F2
F3
TA MDX
MyBP-C fast
Obscurin RhoGEF