Myosin Light Chain Kinase Is Central to Smooth Muscle Contraction and Required for Gastrointestinal Motility in Mice

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Background & Aims: **Smooth muscle is essential for maintaining homeostasis for many body functions and provides adaptive responses to stresses imposed by pathologic disorders. Identified cell signaling networks have defined many potential mechanisms for initiating smooth muscle contraction with or without myosin regulatory light chain (RLC) phosphorylation by myosin light chain kinase (MLCK). We generated tamoxifen-inducible and smooth muscle–specific MLCK knockout (KO) mice and provide direct loss-of-function evidence that shows the primary importance of MLCK in phasic smooth muscle contractions.** ***Methods:*** **We used the Cre-*loxP* system to establish *Mlck* floxed mice in which exons 23, 24, and 25 were flanked by 2 *lox*P sites. Smooth muscle–specific MLCK KO mice were generated by crossing *Mlck* floxed mice with SM-CreER^{T2} (ki) mice followed by tamoxifen treatment. The phenotype was assessed by histologic, biochemical, molecular, cell biological, and physiologic analyses.** ***Results:*** **Targeted deletion of MLCK in adult mouse smooth muscle resulted in severe gut dysmotility characterized by weak peristalsis, dilation of the digestive tract, and reduction of feces excretion and food intake. There was also abnormal urinary bladder function and lower blood pressure. Isolated muscles showed a loss of RLC phosphorylation and force development induced by K^+-depolarization. The kinase knockout also markedly reduced RLC phosphorylation and force development with acetylcholine which activates Ca^{2+}-sensitizing signaling pathways.** ***Conclusions:*** **MLCK and its phosphorylation of RLC are required physiologically for smooth muscle contraction and are essential for normal gastrointestinal motility.**

Smooth muscles are responsible for contraction of the hollow organs in the body such as the gastrointestinal tract, urinary bladder, blood vessels, and the uterus. Normal contractility is essential for maintaining homeostasis and adaptive responses to stresses imposed by pathologic disorders. Smooth muscle contractility is regulated by a network of signaling pathways centered on the molecular motor myosin as well as membrane properties associated with calcium handling and cell adhesion.[1–5] Depolarization of the cell membrane activates voltage-gated Ca^{2+} channels, resulting in Ca^{2+} influx and activation of myosin cross-bridge cycling on actin filaments by regulatory light chain (RLC) phosphorylation catalyzed by Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK). However, agonist stimulation of G protein–coupled receptors on smooth muscle cell surfaces may recruit other regulatory elements. One scheme for gastrointestinal smooth muscle is that the initial increase in $[Ca^{2+}]_i$ is rapidly dissipated, resulting in MLCK inactivation.[3] Maintenance of RLC phosphorylation and muscle force are then regulated by several signaling pathways involving Ca^{2+}-independent kinase(s) and inhibition of myosin phosphatase. There is also evidence that force maintenance switches to thin-filament regulation independent of RLC phosphorylation after the initial increase in $[Ca^{2+}]_i$ when unphosphorylated RLC does not completely inhibit myosin adenosine triphosphatase activity.[6,7]

These conflicting proposals depend largely on results obtained by correlating changes in measured responses such as $[Ca^{2+}]_i$, extents of protein phosphorylations, and force development with interventions by chemical inhibitors in isolated muscles or cells in culture. However, elucidation of MLCK function in vivo is crucial for understanding the complex regulatory processes involved in smooth muscle contraction. Smooth muscle MLCK containing a kinase catalytic core with substrate binding

Abbreviations used in this paper: ACh, acetylcholine; Adv-MLCK, MLCK-expressing adenovirus; BAC, bacterial artificial chromosome; CTR, control; ES, embryonic stem; GFP, green fluorescent protein; IAS, internal anal sphincter; ILK, integrin-linked kinase; KO, knockout; MLCK, myosin light chain kinase; MYPT1, myosin phosphatase protein targeting subunit of the RLC phosphatase; RLC, regulatory light chain; ROCK1, Rho-associated coiled-coil–forming protein kinase 1; RT-PCR, reverse transcription–polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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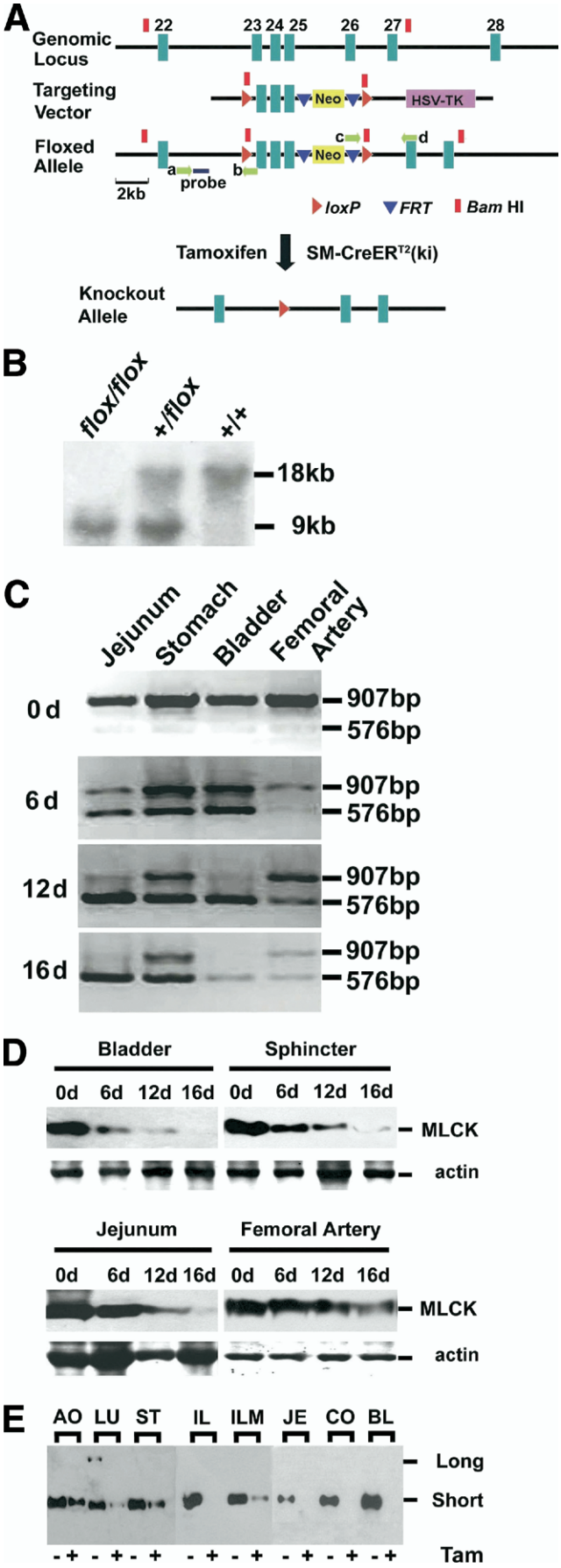

sites and several structural motifs is ubiquitously expressed in different cells in the body.[8–11] Conventional deletion of MLCK expression leads to embryonic or perinatal lethality,[12] making it impossible to determine the functional importance of MLCK in contraction of mature smooth muscle. Thus, we crossed mice containing floxed MLCK alleles with SM-CreER^{T2} (ki) mice expressing a tamoxifen-activated Cre recombinase driven by the SM-22 promoter to delete MLCK expression specifically in smooth muscles of adult mice.

Materials and Methods

Generation of Floxed Mlck Mice and Tissue-Specific Knockout Mice (MLCK^{SMKO})

BAC (bacterial artificial chromosome)–retrieval methods were used for constructing the targeting vector.[13,14] In brief, the *Mlck* locus encoding a portion of the kinase domain was retrieved from a 129/sv BAC clone bMQ 366n04 (provided by Sanger Institute) by a retrieval vector containing 2 homologous arms. Exons 23–25, which encode the adenosine triphosphate–binding site of the kinase, were flanked by 2 *loxP* sites and an *frt-Neo-frt* cassette as a positive selection marker. In addition, this deletion causes an out-of-frame reading shift and thereby generates a premature stop codon and a loss-of-function allele (Figure 1).

Figure 1. Targeted disruption of *Mlck* gene in smooth muscle. (*A*) Schematic representation of *Mlck* smooth muscle–specific knockout strategy. The 8.9-kb genomic DNA fragment containing *Mlck* exons 23–25 was subcloned from 129/sv BAC using gap repair. The floxed *Neo* cassette was targeted upstream of exon 23, and excision of the floxed *Neo* cassette left behind a single *loxP* site (*arrowheads in red*) at the targeted locus. The single PGK-*Neo* cassette flanked by *FRT* sites (*arrowheads in blue*) and a downstream *loxP* site, was then introduced downstream of exon 25. The *Neo* cassette contains a *Bam*HI site (*blocks in red*) that is favorable for Southern blot analysis. The floxed allele (*Mlck^{flox}*) was formed after homologous recombination in ES cells. Mice containing the floxed allele were crossed with SM-CreER^{T2} (ki) mice that express a tamoxifen-activated Cre recombinase to generate *Mlck$^{+/flox}$; SM-CreER^{T2}* and *Mlck$^{flox/flox}$; SM-CreER^{T2}* mice. The ablation of exons 23–25 was induced by tamoxifen injection. The probe used for Southern blot analysis is shown as a *solid blue bar*, and the locations of the PCR primers a–d are indicated by *green arrows*. (*B*) Tail DNA isolated from homozygous (*flox/flox*) floxed, heterozygous (*+/flox*), and wild-type (+/+) mice was digested with *Bam*HI and analyzed by Southern blot. The wild-type and floxed allele yield 18-kb and 9-kb fragments, respectively. (*C*) RT-PCR assay for MLCK mRNA. Various smooth muscle tissues were collected from MLCK^{SMKO} mice induced with tamoxifen at different time points. The mRNA containing exons 23–25 was amplified by RT-PCR. The products in size of 907 bp and 576 bp reflect wild-type and mutated MLCK, respectively. (*D*) Western blots of MLCK in tamoxifen-treated tissues were collected at indicated days (d). Total actin stained with Coomassie Brilliant Blue G-250 was used as protein loading control. Tissues included the urinary bladder, internal anal sphincter, jejunum, and femoral artery. (*E*) Western blots of MLCK protein from floxed mice treated with tamoxifen (Tam+) or its vehicle (Tam-) for 16 days. The amount of loaded protein was normalized by total actin control. AO, aorta; LU, lung; ST, stomach; IL, ileum; ILM, ileum mucosa; JE, jejunum; CO, colon; BL, bladder.

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