Unzipping the Role of Myosin Light Chain Phosphatase in Smooth Muscle Cell Relaxation*

Qi Quan Huang‡, Steven A. Fisher‡§, and Frank V. Brozovich‡¶§

From the Departments of ‡Physiology and Biophysics and §Medicine (Cardiology), Case Western Reserve University, Cleveland, Ohio 44106

Recently, it has been hypothesized that myosin light chain (MLC) phosphatase is activated by cGMP-dependent protein kinase (PKG) via a leucine zipper-leucine zipper (LZ-LZ) interaction through the C-terminal LZ in the myosin-binding subunit (MBS) of MLC phosphatase and the N-terminal LZ of PKG (Surks, H. K., Mochizuki, N., Kasai, Y., Georgescu, S. P., Tang, K. M., Ito, M., Lincoln, T. M., and Mendelsohn, M. E. (1999) Science 286, 1583–1587). Alternative splicing of a 3'-exon produces a LZ* or LZ* MBS, and the sensitivity to cGMP-mediated smooth muscle relaxation correlates with the relative expression of LZ*/LZ* MBS isoforms (Khatri, J. J., Joyce, K. M., Brozovich, F. V., and Fisher, S. A. (2001) J. Biol. Chem. 276, 37250–37257). In the present study, we determined the effect of LZ*/LZ* MBS isoforms on cGMP-induced MLC20 dephosphorylation. Four avian smooth muscle MBS-recombinant adenoviruses were prepared and transfected into cultured embryonic chicken gizzard smooth muscle cells. The expressed exogenous MBS isoforms were shown to replace the endogenous isoform in the MLC phosphatase holoenzyme. The interaction of type I PKG (PKG1) with the MBS did not depend on the presence of cGMP or the MBS LZ. However, direct activation of PKG1 by 8-bromo-cGMP produced a dose-dependent decrease in MLC20 phosphorylation (p < 0.05) only in smooth muscle cells expressing a LZ* MBS. These results suggest that the activation of MLC phosphatase by PKG1 requires a LZ* MBS, but the binding of PKG1 to the MBS is not mediated by a LZ-LZ interaction. Thus, the relative expression of LZ*/LZ* MBS isoforms could explain differences in tissue sensitivity to NO-mediated vasodilation.

Force regulation in smooth muscle is dependent on the activities of myosin light chain (MLC) kinase and MLC phosphatase (3, 4). The activity of MLC kinase is regulated by Ca2+ and calmodulin (3), whereas MLC phosphatase was originally thought to be constitutively active and unregulated (5). However, there is abundant evidence that the activity of MLC phosphatase can be both inhibited to produce Ca2+ sensitization (reviewed in Refs. 5–7) or an increase in force at a constant [Ca2+] and enhanced to produce Ca2+ desensitization (8) or a decrease in force at a constant [Ca2+]. NO is the classical agent to produce Ca2+ desensitization (1, 9, 10). Recent evidence (11) suggests that NO produces vasodilation by activating the soluble pool of guanylate cyclase, which in turn produces cGMP and leads to the activation of type I cGMP-dependent protein kinase (PKG1). PKG1 mediates smooth muscle cell relaxation by several mechanisms. It has been demonstrated that PKG1 acts on the maxi K+ channel to produce hyperpolarization of the smooth muscle (12), decreases Ca2+ flux (13, 14), and also activates MLC phosphatase (1, 15) to decrease the level of MLC20 phosphorylation and to produce smooth muscle relaxation. In addition, PKG1-dependent pathways for vasodilation may also include phosphorylation of telokin (16, 17) and HSP20 (18).

MLC phosphatase is a holoenzyme consisting of a catalytic subunit (PP1cδ); a myosin-binding subunit (MBS), which is also referred to as the myosin-targeting subunit (MYPT1); and a 20-kDa subunit of unknown function (5). The activation of MLC phosphatase by PKG1 is hypothesized to be due to a leucine zipper-leucine zipper (LZ-LZ) interaction of the N-terminal LZ of PKG1α and the C-terminal LZ of the MBS of MLC phosphatase (1, 2). The MBS has four major isoforms, which are produced by alternative RNA splicing of two different exons (5). Tissue-specific and developmentally regulated alternative splicing of a 123-bp central exon produces a 41-amino acid central insert (19). Alternative splicing of the 31-bp 3'-exon is responsible for the expression of LZ* or LZ* MBS isoforms (5). Specifically, exclusion of the 3'-exon shifts the reading frame of the MBS transcript to encode a C-terminal LZ (2). We previously demonstrated that sensitivity to cGMP-mediated relaxation correlates with the relative expression of LZ*/LZ* MBS isoforms (2), which is consistent with the activation of MLC phosphatase activity resulting from a LZ-LZ interaction of PKG1α with the MBS (1). In this study, we tested the hypothesis that cGMP-dependent activation of MLC phosphatase activity and smooth muscle vasodilation are due to a LZ-LZ interaction of PKG1α and MBS by changing the expression of the MBS isoform, in isolation, and determining the effect on cGMP-mediated MLC20 dephosphorylation in primary cultured smooth muscle cells (SMCs).

**MATERIALS AND METHODS**

Cloning of the Chicken MBS of the MLC Phosphatase cDNA Fragment—Total RNA was isolated from fresh chicken gizzard and urinary bladder smooth muscle tissues using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Full-length cDNA encoding the MBS was synthesized by reverse transcription-PCR using the total RNA as template. We used primers 5'-GGGGCAATATGGCAGGAGGTTTACACAG-3' and 5'-CAGGTAAGAGGGCATTTGGCAGGATA-3', flanking the MBS cDNA sequence between bp +86 and +3382 (20). The PCR

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products were cloned into the pCR2.1 plasmid vector (Invitrogen). cDNAs corresponding to four alternative splicing variants of the chicken smooth muscle MBS, differing in the inclusion or exclusion of a central exon and a 3' exon, were screened (see Fig. 1) and confirmed by DNA sequencing. Note that the sequence at base 1822 is C instead of G, which encodes position 568; the GTT (valine) reported by Shimizu et al. (20) is CTT (leucine) in eight independent clones obtained by reverse transcription-PCR.

**Construction of Recombinant Adenoviruses Encoding MBS Isoforms**—Recombinant adenoviruses were generated using the AdEasy system (21). The four MBS cDNAs were individually cloned into transfer vector pAdTrack-CMV (9.2 kb, kanamycin-resistant) downstream of a cytomegalovirus promoter. This vector has a second cytomegalovirus promoter expressing green fluorescent protein. *Escherichia coli* B35183 cells were first transformed with supercoiled adenoviral backbone DNA pAdEasy-1 (33.4 kb, ampicillin-resistant). The transformants were confirmed by restriction enzyme mapping and then prepared in competent cells by an acid salt method (22). For *in vivo* homologous recombination in the bacterial cell, ~0.5–1 µg of linearized MBS-pAdTrack (12.5 kb) recombinant plasmid DNA was transformed into the above competent *E. coli* cells, followed by plating on LB agar medium containing 50 µg/ml kanamycin. The MBS-recombinant adenovirus plasmids were retransformed into a *recA* strain of *E. coli* (JM109) for large-scale DNA preparation and verified by restriction enzyme mapping and PCR using two pairs of specific MBS primers: 5’-1620/3’-2069 (flanking the central alternative exon) and 5’-2888/3’-3203 (flanking the 3'-end alternative exon), respectively (see Fig. 1). To produce the MBS-recombinant adenovirus, 293 mammalian packaging cells (Quantum Biotechnology) were grown to 70% confluence in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, 100 µg/ml penicillin, and 50 µg/ml streptomycin. 5 µg of each MBS-recombinant adenovirus plasmid DNA was linearized by the restriction enzyme PacI and used for transfection of 293 cells with LipofectAMINE™ 2000 reagent (Invitrogen) according to the manufacturer’s protocol. The amplified MBS-recombinant viruses and their protein products in 293 cells were verified by PCR as described above and by Western blotting using anti-MYPT1 antibody.

**Fig. 1. Structure of the alternatively spliced chicken MBS cDNA and verification of the recombinant plasmids and viruses.** 

A. Shown is the structure of the 3.3-kb chicken MBS cDNA. The positions of the two alternatively spliced exons, two pairs of oligonucleotide primers used in this study, and three internal plus one flanking PstI restriction enzyme-cutting sites are indicated with the base numbers. B. Recombinant plasmids bearing MBS cDNAs including or excluding either of the alternative exons are named as In/In, In/out, Out/In, Out/out, respectively, and were verified by their distinct PstI restriction patterns (left panel) and the different sizes of the specific PCR fragments (right panel). C. The proteins encoded by the cDNAs were verified by their gel mobility upon 6% SDS-PAGE and Western blotting using anti-MBS antibody. The four transgenes produced four distinct isoforms of MBS. Their predicted sizes are as follows: CI+AZL+, 111 kDa; CI+AZL−, 115 kDa; CI−AZL+, 107 kDa; and CI−AZL−, 110. Ad, adenovirus; MCS, multiple cloning site.
(Upstate Biotechnology, Inc.), respectively (Fig. 1). After verification, a few rounds of amplification were performed in 293 cells to produce high titer viral stocks. All four MBS-recombinant adenoviruses and an adenovirus vector control were titrated by plaque forming units in 293 cell cultures.

Preparation of Chicken Smooth Muscle Cells—Primary cultures of chicken gizzard SMCs were isolated from day 15 embryos using a modification of a previously described method (23, 24). Briefly, after being minced into fine pieces, the gizzard tissue was incubated in Hanks’ balanced salt solution (Cellgro) containing 0.15% (w/v) collagenase type I ( Worthington) at 37 °C for 20–40 min. Dispersed single cells were collected by passing the cell suspension through a cell strainer (70 μm; BD Biosciences). The cells were then collected by centrifugation at 2000 × g for 5 min and washed twice with cell culture medium (50:50 mixture of Dulbecco’s modified Eagle’s medium and nutrient mixture P-12 medium plus 0.5% fetal bovine serum, 10 mM sodium molybdate, 5% (v/v) streptomycin). The cells were suspended in cell culture medium and plated in culture dishes at 3 °C for 2 h. The infected cell cultures were then preincubated with 10% (v/v) FBS and 10% (v/v) normal rabbit serum as a negative control. After rotating the lysate/protein G-Sepharose beads were collected, suspended in 50 μl of SDS sample buffer, and analyzed by SDS-PAGE and Western blotting as described above.

Western Blot Analyses—Total protein was extracted from cultured SMCs or chicken tissue samples in SDS sample buffer and resolved by SDS-PAGE at an acrylamide/bisacrylamide ratio of 29:1. MBS was cleaned by passing through an Ultrafree-MC centrifugal filter (Amicon, Inc.) at 12,000 × g for 5 min. Non-phosphorylated MLC20, monophos-
phosphorylated MLC\textsubscript{20} (P\textsubscript{1}-MLC\textsubscript{20}), and diphosphorylated MLC\textsubscript{20} (P\textsubscript{2}-MLC\textsubscript{20}), were resolved by electrophoresis using a urea/glycerol-PAGE system with 10% polyacrylamide (acrylamide/bisacrylamide ratio of 19:1), 40% (v/v) glycerol, 22 mM Tris-HCl (pH 8.6), and 22 mM glycine (26, 27), running at 300 V for 5–6 h in buffer containing 22 mM Tris-HCl, 22 mM glycine, 10 mM dithiothreitol, and 0.1% thioglycolic acid (pH 8.6). The gel was then transferred to nitrocellulose membrane for Western blotting as described above. MLC\textsubscript{20} bands were identified using anti-MLC\textsubscript{20} antibody and quantified by Scion Image Beta Version 4.0.2 software. The percentage of phosphorylated MLC\textsubscript{20} was calculated by the ratio of phosphorylated MLC\textsubscript{20} to total MLC\textsubscript{20} (P\textsubscript{1}-MLC\textsubscript{20} or P\textsubscript{2}-MLC\textsubscript{20}/(P\textsubscript{1}-MLC\textsubscript{20} + P\textsubscript{2}-MLC\textsubscript{20} + MLC\textsubscript{20})). The data are expressed as a percentage of phosphorylated MLC\textsubscript{20} (P\textsubscript{1}-MLC\textsubscript{20} or P\textsubscript{2}-MLC\textsubscript{20}) against unstimulated controls. All values are means ± S.D., and \( p < 0.05 \) was taken as the level for significance.

RESULTS

The full-length chicken smooth muscle MBS cDNA is 4.7 kb, with the coding region from base 121 to 3251 (20). Alternative RNA splicing of a central exon and the 3′-exon produces several different protein isoforms, and the expression of these isoforms is both developmentally regulated and tissue-specific (2, 19). The MBS isoforms differ by the presence or absence of the central insert (CI\textsuperscript{+} or CI\textsuperscript{−}) and the C-terminal LZ (LZ\textsuperscript{+} or LZ\textsuperscript{−}). In this study, four distinct cDNAs encoding the CI\textsuperscript{+}LZ\textsuperscript{+}, CI\textsuperscript{−}LZ\textsuperscript{−}, CI\textsuperscript{−}LZ\textsuperscript{+} and CI\textsuperscript{+}LZ\textsuperscript{−} isoforms, respectively, were cloned and successfully expressed in both a mammalian cell line and primary cultured avian SMCs.

It has been well documented that primary cultured SMCs under conventional serum-stimulated conditions quickly convert from a differentiated to dedifferentiated state. The degree of dedifferentiation is related to the time and passage in culture (28), and it has been documented that the expression of PKG is rapidly down-regulated with the passage of SMCs (29).

In our experiments, the number of freshly isolated single cells obtained during isolation from the tissue was sufficient, so the cells did not need to be passaged; and the chicken gizzard SMCs were always kept in low serum (0.5%) medium to maintain a differentiated state. The SMCs for these conditions expressed ~60% of the PKG compared with tissue at this stage and markers of the differentiated state such as calponin and smooth muscle myosin light chain and heavy chain (data not shown).

Recombinant adenoviruses are currently used for a variety of purposes, including gene transfer. We chose this technique as the delivery vehicle to bring the MBS expression construct into SMCs because it has a high transfection efficiency, and SMCs do not need to be passaged for transfection. Normally, 60–100% of the cells are infected. SMCs infected by recombinant adenovirus express predominantly the expected exogenous MBS isoform. The exogenous MBS was overexpressed at levels 5–10 times higher than the endogenous MBS (Fig. 2), without changing the level of expression of other major smooth muscle proteins, including PKGI (both \( \alpha \) and \( \beta \) isoforms), calponin, PP1c\( \delta \), and MLC\textsubscript{20}, compared with the uninfected control SMCs or SMCs infected with an adenovirus vector alone (Fig. 2).

Co-immunoprecipitation assays using anti-PP1c\( \delta \) antibody were performed to capture PP1c\( \delta \) from the lysate of SMCs overexpressing a single MBS isoform to determine whether the exogenous MBS replaced the endogenous isoform of MBS in the MLC phosphatase holoenzyme. The results show that the immunoprecipitates of PP1c\( \delta \) in all groups of the cell lysates contained MBS isoform patterns similar to those in the corresponding whole cell lysate (Fig. 3A), indicating that the overexpressed exogenous MBS isoform was proportionally assembled into the MLC phosphatase complex to replace the endogenous MBS isoform in the holoenzyme.

Co-immunoprecipitation by anti-PKG\( \alpha \) antibody was used to determine whether PKGI was associated with the MBS. Our results demonstrate that, in cultured SMCs, neither the LZ\textsuperscript{+} nor LZ\textsuperscript{−} MBS isoforms bound to PKGI (Fig. 3B). This contrasts with the results obtained with both embryonic and day 7 chicken aortic and gizzard tissues, where we found that both LZ\textsuperscript{+} and LZ\textsuperscript{−} MBS isoforms associated with PKGI, and although the association did not depend on the presence of cGMP, cGMP appeared to increase the binding of the MBS to PKGI in the aorta (Fig. 3C). Comparing adult gizzard, aorta, and SMCs, the PKG/MBS ratio was highest in adult gizzard and lowest in cultured SMCs.

It has been reported that the interaction of the N-terminal LZ of PKGI\( \alpha \) (but not PKGI\( \beta \)) mediates cGMP-stimulated smooth muscle relaxation through interaction with the C-terminal LZ of the MBS of MLC phosphatase (1). We used both reverse transcription-PCR with PKGI\( \alpha \) or PKGI\( \beta \) isof orm-specific primers and Western blotting with both a PKGI\( \alpha \)-specific antibody and a PKGI\( \beta \)-specific antibody to show the expression of PKGI\( \alpha \) versus PKGI\( \beta \) in embryonic and adult chicken aortic and gizzard smooth muscle tissues as well as in cultured SMCs (Fig. 4). Expression of PKGI in embryonic day 15 chicken aortic and gizzard smooth muscle tissues was similar; and after 72 h in culture, PKGI expression in the SMCs...
was 50–60% of the level at embryonic day 15. In addition, the expression of PKGI was up-regulated in aortic and down-regulated in gizzard smooth muscle tissues during development and after hatching (Fig. 4). The Western blots confirm that, in the smooth muscle tissue, only PKGI was expressed (Fig. 4). In cultured SMCs, PKGI expression predominated, as PKGI was barely detected (Fig. 4).

MLC phosphorylation levels were determined after stimulation of infected monolayer cultures of SMCs with 8-Br-cGMP. The control cultured SMCs expressed an endogenous MBS isoform that was CI and LZ. 8-Br-cGMP stimulation resulted in a dose-dependent decrease in the level of phosphorylation of MLC20 (P2-MLC20) in SMCs expressing a LZ MBS (Fig. 5). In uninfected control SMCs as well as in SMCs infected with adenovirus vector or MBS-recombinant adenoviruses expressing an exogenous LZ MBS, the level of P2-MLC20 decreased to ~65% of the unstimulated control at 0.01–1 mM 8-Br-cGMP. However, in cells overexpressing LZ MBS isoforms, 8-Br-cGMP produced a significant decrease in MLC phosphorylation only at the highest concentration of 8-Br-cGMP (10−3 M) (Fig. 5). These results suggest that the expression of the C-terminal LZ of the MBS may be required for PKG to activate MLC phosphatase during cGMP-mediated smooth muscle relaxation.

**DISCUSSION**

In this study, we have shown that we could change the expression of the MBS isoform of the MLC phosphatase, in isolation, without changing the expression of other contractile proteins (Fig. 2), and the exogenous MBS isoform replaced the endogenous isoform in the MLC phosphatase holoenzyme (Fig. 3A). Our data show that cGMP stimulation of MLC phosphatase containing a LZ MBS isoform produced a dose-dependent decrease in MLC20 phosphorylation, whereas cGMP stimulation of MLC phosphatase containing LZ MBS isoforms did not produce dose-dependent dephosphorylation of MLC20 (Fig. 5). These data demonstrate that, in cultured SMCs, a LZ MBS is required for PKG activation of MLC phosphatase activity.

Previous studies have suggested that the activation of MLC phosphatase by PKG is due to a LZ-LZ interaction of PKGI and the MBS (1). A series of PKG constructs were used to show that only PKG with the LZ would co-immunoprecipitate with the MBS. Our co-immunoprecipitation studies with the cultured chicken SMCs did not detect an association of either LZ or LZ MBS isoforms with PKG. However, in both embryonic and adult smooth muscle tissues, we observed the association of both LZ (aortic) and LZ (adult gizzard) MBS isoforms with PKG. However, in both embryonic and adult smooth muscle tissues, we observed the association of both LZ (aortic) and LZ (adult gizzard) MBS isoforms with PKG. In this study (2), we could not co-immunoprecipitate the LZ MBS from adult chicken gizzard, possibly because the expression of PKG was rapidly down-regulated after hatching (Fig. 4). These results suggest that a LZ-LZ interaction is not the sole determinant for the association of PKG and the MBS.
interaction does not mediate the interaction of PKG with the MBS.

It is unclear why we did not observe association of the MBS and PKG in cultured SMCs, whereas the MBS and PKG were associated in both the presence and absence of cGMP in chicken tissue expressing either LZ/H11001 or LZ/H11002 MBS isoforms. However, in the cultured SMCs, we can rule out that the absence of an association of the MBS with PKG is due to a lack of PKGI/H9251 expression (Fig. 4). Our data show that an association between PKG and the MBS is not required for cGMP to activate MLC phosphatase activity, although a LZ/H11001 MBS isoform is necessary for cGMP to stimulate MLC20 dephosphorylation. PKG has multiple targets in smooth muscle (1, 13, 14, 15), and a cofactor or an anchoring protein maybe required for PKG to interact with or target to the MBS. Our data suggest that an association of PKG and the MBS is needed for PKG to efficiently activate MLC phosphatase activity, and the lack of this interaction in cultured SMCs could explain the difference in sensitivity to cGMP in chicken tissue with a LZ/H11001 MBS, where force relaxation is seen at 0.1 μM 8-Br-cGMP (2), and in

**FIG. 5. MLC20 phosphorylation in response to 8-Br-cGMP stimulation.** MLC20 phosphorylation in SMCs was examined in response to 10⁻³ to 10⁻⁶ M 8-Br-cGMP stimulation. SMCs differing in the overexpressed exogenous MBS isoform were compared with control cells and empty adenovirus vector (Ad vector control)-infected cells, both expressing the endogenous LZ isoform of MBS. A, MLC20 (non-phosphorylated), P1-MLC20, and P2-MLC20 were resolved by urea/glycerol-PAGE and examined by Western blotting using anti-MLC20 antibody. Two samples of SDS gel patterns are displayed, and the three MLC20 bands are indicated. B, shown are the P2-MLC20 levels versus [8-Br-cGMP]. Results are expressed as the percentage of P2-MLC20 normalized to the unstimulated control (100%).
cultured SMCs expressing LZ
MBS isoforms, where MLC
dephosphorylation begins between 1 and 10 µM 8-Br-cGMP (Fig. 5). However, both in our previous report performed with smooth muscle strips (2) and in the present study of cultured SMCs, a LZ
MBS is required for cGMP-mediated smooth muscle relaxation.

Taken together, these results suggest that the interaction of PKG with the MBS is not due to a LZ-LZ interaction, but could be due to an interaction of a coiled-coil domain of PKG with the coiled-coil domain of the MBS. The MBS has a predicted coiled-coil domain between amino acids 647–705 and amino acids 888–928 (30); and similarly, the N-terminal 10–46 residues of PKGI have a 100% probability of a coiled-coil domain structure. It should be noted that all of the constructs of PKG used by Surks et al. (1) containing the LZ also contained the coiled-coil domain, and mutations that disrupted the coiled-coil domain of PKG inhibited binding of PKG and the MBS. Thus, the results of Surks et al. could also be consistent with an interaction of the coiled-coil domain of PKG with the coiled-coil domain of the MBS.

The mechanism for activation of MLC phosphatase by PKG is unknown. It is possible that PKG and the MBS interact via coiled-coil domains, and activation of MLC phosphatase activity could be due to phosphorylation of the MBS by PKG. It has been reported that PKG phosphorylates the MBS (1). PKG binding to substrates is mediated by an Arg-Arg or Arg-Lys sequence (31), and PKG phosphorylates at a Ser-Ser sequence (32). There are consensus sites for PKG binding at residues 841 and 842 (Arg-Arg) and residues 847 and 848 (Arg-Arg), and a Ser-Phosphorylation site lies at residues 790–794 of the MBS, which are of between the two predicted coiled-coil domains of the MBS. Another PKG-binding site can be found at residues 916 and 917 (Arg-Lys); and similarly, another Ser-Phosphorylation sequence lies at residues 895 and 896 of the MBS, which are within one of the predicted coiled-coil domains (amino acids 888–928) and ~100 residues from the beginning of the C-terminal LZ (amino acid 1002). It could be that the presence of the C-terminal LZ in the MBS exposes the more C-terminal binding and phosphorylation sites to PKG and allows for PKG to bind to and phosphorylate the MBS and thus to increase MLC phosphatase activity. In LZ
MBS isoforms, PKG could bind to the MBS, but the phosphorylation sites may not be accessible to PKG, and MLC phosphatase with a LZ
MBS would not be activated by PKG.

In summary, the results of this study suggest that PKG binds to the MBS, possibly via coiled-coil domain interactions, and the activation of MLC phosphatase activity by PKG requires an LZ
MBS. The expression of LZ
MBS isoforms is developmentally regulated and tissue-specific (2); and thus, the tissue diversity in the sensitivity to NO-mediated vasodilation in smooth muscle could be determined, in part, by the relative expression of LZ
LZ
MBS isoforms.

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