Identification and Characterization of Zipper-interacting Protein Kinase as the Unique Vascular Smooth Muscle Myosin Phosphatase-associated Kinase*

Received for publication, April 2, 2004, and in revised form, July 13, 2004
Published, JBC Papers in Press, July 30, 2004, DOI 10.1074/jbc.M403676200

Akira Endo‡, Howard K. Surks§, Seibu Mochizuki, Naoki Mochizuki¶, and Michael E. Mendelsohn‡‡
From the ‡Molecular Cardiology Research Institute, New England Medical Center and Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts, the §Division of Cardiology, Department of Internal Medicine, Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan, and the ¶Department of Structural Analysis, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan

Excitation-contraction coupling in smooth muscle involves activation of myosin light chain (MLC) phosphorylation, which increases activity of the myosin actin-activated ATPase, resulting in contraction. Phosphorylation of MLC phosphatase (SMPP-1M) by Rho-associated kinase or endogenous SMPP-1M-associated kinase inhibits SMPP-1M, enhancing MLC phosphorylation and contraction. However, the precise identity of SMPP-1M-associated kinase remains unclear. Biochemical evidence strongly supports the idea that SMPP-1M-associated kinase is related to the human serine/threonine leucine zipper-interacting protein kinase (ZIPK), which is important in cell apoptosis, and the SMPP-1M-associated kinase has therefore been called ZIP-like kinase (MacDonald, J. A., Borman, M. A., Murani, A., Somlyo, A. V., Hartshorne, D. J., and Haystead, T. A. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2419–2424). Whether the vascular smooth muscle SMPP-1M-associated kinase is a truncated version of hZIPK, native hZIPK, or a unique homologue of hZIPK is unclear. Here we show that only native hZIPK mRNA and protein are detectable in human vascular smooth muscle cells (VSMCs). High stringency screening of a human aortic cDNA library for the SMPP-1M-associated kinase identified 18 positive clones, all of which proved to be clones of hZIPK. PCR-based studies of VSMC RNA revealed native hZIPK transcripts but no evidence for splice variants of hZIPK or a ZIP-like kinase. Northern blotting studies of multiple vascular and non-vascular tissue RNAs, including human bladder RNA, showed only 2.3 kb of mRNA predicted for full-length hZIPK. Immunoblotting showed native full-length 52-kDa hZIPK expression in VSMCs. Full-length and N-terminal hZIPK bound the C-terminal domain (amino acids 681–847) of the myosin binding subunit (MBS) of SMPP-1M. hZIPK immunoprecipitated with the MBS of SMPP-1M and dominant negative RhoA inhibited the hZIPK-MBS interaction. These data identify hZIPK as the unique SMPP-1-associated kinase expressed in human vascular smooth muscle and support a role for Rho in promoting the hZIPK-MBS interaction.

Blood vessel tone regulates blood pressure and flow and is itself dynamically regulated by the contractile state of vascular smooth muscle cells (VSMCs) in the blood vessel wall. Contraction and relaxation of VSMCs is determined by the phosphorylation state of myosin light chains (MLCs), a process that is tightly regulated by the opposing activities of myosin light chain kinase and myosin phosphatase (SMPP-1M) (1, 2). Myosin phosphatase is the critical enzyme that dephosphorylates MLC, leading to cell relaxation (3).

In recent years, accumulating evidence supports the view that myosin phosphatase activity is highly regulated. Nitrovasodilators, via cGMP and cGMP-dependent protein kinase Iα, lead to activation of PP1M and cell relaxation (4–8). Vasoconstrictors, conversely, increase MLC phosphorylation by at least two pathways, namely activation of MLCK (9, 10) and inhibition of SMPP-1M. Vasoconstrictors can inhibit SMPP-1M by activation of the potent PP1M inhibitor CPI-17 (11, 12) or via RhoA-mediated SMPP-1M phosphorylation (13). RhoA, when activated by vasoconstrictors, binds and activates its effector Rho-kinase, which leads to SMPP-1M phosphorylation (13). Although the mechanism by which RhoA and Rho-kinase are targeted to SMPP-1M on contractile fibers is unclear, both RhoA and SMPP-1M have been shown recently to interact with the actin-binding protein M-RIP (14).

Biochemical isolation of SMPP-1M has lead to the recovery of SMPP-1M-associated kinase activity (15–19). The SMPP-1M-associated kinase phosphorylates the myosin binding subunit (MBS) of SMPP-1M and inhibits SMPP-1M activity. As recent data support the physiologic importance of SMPP-1M regulation, attention has focused on identification of the SMPP-1M-associated kinase(s).

Recently, MacDonald et al. identified a SMPP-1M-associated kinase and named it ZIP-like kinase. ZIP-like kinase was isolated from bladder smooth muscle as a 32-Da phosphoprotein that co-purified with SMPP-1M, phosphorylated SMPP-1M at inhibitory residues, and was activated by the smooth muscle contractile agonist carbachol (16). Furthermore, introduction of ZIP-like kinase into rabbit ileal smooth muscle led to calcium-independent contractions (20).

Despite data supporting a physiological role for a ZIP-like kinase in smooth muscle, the precise identity of this kinase and
its presence and function in VSMCs remains unclear. Sequenc-
ing of ZIP-like kinase-derived peptides revealed high homology to the 52-kDa zipper-interacting protein kinase (hZIPK), rais-
ing the possibility that ZIP-like kinase could be a splice variant of hZIPK, a separate kinase with high homology to hZIPK, or a degradation product of hZIPK (16). hZIPK was identified originally as a protein involved in programmed cell death that interacts with the death-induction factor ATF4 and mediates apopto-
asis when overexpressed (21). hZIPK has also been shown to have a potential role in regulating VSMC contraction. hZIPK was found to phosphorylate MLC at both Ser^18 and Thr^18 in a calcium-independent manner, leading to cell contraction (22).

The following study was initiated to determine the precise identity of the PP1-M-associated kinase(s) in VSMCs. Through a combina-
tion of mRNA and protein analysis, we have found evidence to strongly support the proposition that "ZIP-like kinase" in VSMCs is actually derived from hZIPK, which is a splice variant of ZIPK-like kinase (23).

MATERIALS AND METHODS

Antibodies—Sources of antibodies were as follows. The rabbit poly-
clonal anti-ZIP kinase (amino acids 279–296) was from Calbiochem, the anti-FLAG-M2 antibody was from Sigma, the anti-myosin phosphatase polyclonal antibody came from Covance (Berkeley, CA), and normal mouse IgG and normal rabbit IgG were from Santa Cruz Biotechnology Inc., Santa Cruz, CA.

Cell Culture and Transfection—Human embryonic kidney 293 (HEK293) cells were purchased from the American Type Culture Collection (Manassas, VA). Immortalized aorta smooth muscle cells, coronary smooth muscle cells, pulmonary artery smooth muscle cells, and radial artery smooth muscle cells were developed in our laboratory from human tissues by the explant method. These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HEK293 cells were transfected by the calcium phosphate method.

Plasmids—To generate plasmids expressing hZIPK, cDNA frag-
ments were ampliﬁed by a polymerase chain reaction from pACT2 (Clontech) containing hZIPK fragments and cloned into pT7Blue-3 (Nov-
agen, Madison, WI). pT7Blue-hZIPK was digested with EcoRI and BamHI, and the fragments were cloned into the pFLAG-CMV4 vector (Sigma). GST fusion proteins of full-length and C-terminal hZIPK were generated as follows. DNA fragments corresponding to full-length and C-terminal hZIPK were ampliﬁed by PCR using the full-length primers of 5'-GGGATCCATGACGCGGCGCTGCACTCCACGGGCCTGCAG-3' (5') and 5'-GGTCGACCTCGAGCAGGACCGGCGCTGCACTCCACGGGCCTGCAG-3' (3') and the C-terminal hZIPK primers of 5'-GGGATCCATGACGCGGCGCTGCACTCCACGGGCCTGCAG-5' and 5'-GGTCGACCTCGAGCAGGACCGGCGCTGCACTCCACGGGCCTGCAG-3' (5') and 5'-GGTCGACCTCGAGCAGGACCGGCGCTGCACTCCACGGGCCTGCAG-3' (3') for the latter. The products were cloned into pT7Blue-3 and digested with EcoRI and SalI. These fragments were cloned into pGEX4T-3 and pGEX4T-1 vectors (Amer sham Biosciences). To generate the plasmid expressing the GST-N-terminal region of hZIPK, NotI-digested pGEX4T-3 hZIPK was treated with a Klenow fragment (New England Biolabs, Beverly, MA) to blunt the ends, followed by self-ligation. PEF-BOS-mouse ZIPK was a gift from S. Akira, Department of Biochemistry, Yokogawa College of Medicine. pCXN2-ires-EGFP (23) expression vectors were derived from pCAGGS and contain the internal ribosomal entry site (IRES) and the coding region of the enhanced green fluorescent protein (EGFP) at the 3'-end of the multiple cloning site. The DNA ligated EcoRI-NotI frag-
ments of hZIPK cDNA were made from the cDNA clone A1660136 (Genome System) encoding the N terminus of hZIPK and used as a probe. The library was transferred to Hybond N synthetic nylon membranes (Amer sham Biosciences) and then pre-hybridized and hybrid-
ized in 5× Denhardt's solution, 5× SSC, 0.1 M sodium phosphate, 0.5% SDS, 0.1% SDS denatured salmon sperm DNA under high hybridiz-
ation temperature 65°C and low hybridization temperature 58°C (36) stringency conditions.

Polymerase Chain Reaction—PCR was performed in a ﬁnal volume of 50 μl of reaction mix containing 0.2 mM each dNTP, 1.5 mM MgCl2, PCR buffer without magnesium, 0.25 μM each primer for the investigation of ZIPK splice variants, 500 μM each primer for detection of 72 units of Taq DNA polymerase (Invitrogen). Reaction mixtures were heated for 5 min at 95°C, followed by 30 cycles of ampliﬁcation. Each cycle consisted of denaturation at 95°C for 30 s, annealing between 38 and 57°C, and extension at 70°C between 1 and 3 min. After the last cycle, samples were incubated for an additional 10 min at 70°C. The following primers were designed for the investigation of hZIPK splice variants: primer 1, 5'-AGGCCTGGTCTGGGCGGAGGCGG-3' (5'); primer 2, 5'-AGGCGCGTCGGCGCGAGGGGGCGCC-3' (5') and 3'-CCTCAGTCTCAAGTGTGACCCTCC-3'; primer 3, 5'-AGGCGCGTCGGCGCGAGGGGGCGCC-3' (5') and 3'-CCTCAGTCTCAAGTGTGACCCTCC-3'; primer 4, 5'-AGGCGCGTCGGCGCGAGGGGGCGCC-3' (5') and 3'-CCTCAGTCTCAAGTGTGACCCTCC-3'; primer 5, 5'-AGGACATGCTGCACGAGG-3' (5') and 3'-CCTCAGTCTCAAGTGTGACCCTCC-3'; primer 6, 5'-ATGCACGGTTCGGAGGGCGG-3' (5') and 3'-GCTCGGCTTGCGGCGCC-3'; primer 7, 5'-AGGACATGCTGCACGAGG-3' (5') and 3'-CCTCAGTCTCAAGTGTGACCCTCC-3'; primer 8, 5'-AGGACATGCTGCACGAGG-3' (5') and 3'-CCTCAGTCTCAAGTGTGACCCTCC-3'; primer 9, 5'-AGGACATGCTGCACGAGG-3' (5') and 3'-CCTCAGTCTCAAGTGTGACCCTCC-3'; primer 10, 5'-AGGACATGCTGCACGAGG-3' (5') and 3'-CCTCAGTCTCAAGTGTGACCCTCC-3'; primer 11, 5'-AGGACATGCTGCACGAGG-3' (5') and 3'-CCTCAGTCTCAAGTGTGACCCTCC-3'.

The following primers were used for degenerate PCR: primer IA, 5'-ATGGGNGARGARYTNGG-5' (5') and 5'-YTGDATNGNCGGC-3' (5'); primer IB, 5'-ATGGGNGARGARYTNGG-5' (5') and 5'-RRADATTNNYNRTTC-3' (5'); primer IA, 5'-GAYAARNNNAHTTT-5' (5') and 5'-ARRTTYGNGNC-3' (5'); primer IB, 5'-MGNCCNATHC-3' (5') and 5'-ARRTTYGNGNC-3' (5'); primer II, 5'-ATGGGNGARGAYTNGG-5' (5') and 5'-ARRTTYGNGNC-3' (5'); primer III, 5'-ATGGGNGARGAYTNGG-5' (5') and 5'-ARRTTYGNGNC-3' (5'). The primers 5'-ATGGGNGARGARYTNGG-5' (5') and 5'-GAYAARNNNAHTTT-5' (5') and 5'-MGNCCNATHC-3' (5') and 5'-ARRTTYGNGNC-3' (5') corresponded to amino acids 450–460 of the human ZIPK cDNA and a 1.4-kb EcoRI-BamHI fragment encoding full-length human ZIPK were made from pFLAG-CMV4-ZIPK. A 1-kb SalI-NotI fragment encoding the 5'-end of mouse ZIPK and a 1.3-kb SalI-SalI fragment encoding full-length mouse ZIPK were made from pEF-BOS-ZIPK.

Preparation of GST Fusion Proteins for Binding Studies—BL21 cells were transformed with GST-ZIPK plasmids, described above, and GST-MBS plasmids expressing the C-terminal 183 aa of MBS or the C-terminal 183 aa in which the four leucine residues of the leucine zipper domain have been mutated to alanines (L1007A, L1014A, L1021A, and L1026A) were made as described previously (14). The transformed cells were grown at 30°C until the absorbance at 600 nm reached 0.6–0.8. Protein expression was induced by the addition of 0.1% isopropyl-β-thiogalactopyranoside for 3 h, and GST fusion proteins were isolated by using glutathione-Sepharose 4B beads.

Immunoprecipitation, Immunoblotting, and Cell Staining—Cells were washed with ice-cold Tris-buffered saline (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 2 mM EDTA, 10% glycerol, 25 mM β-glycerophosphate, protease inhibitor mixture set III (Calbiochem), and protease inhibitor mixture tablets (Roche Applied Science). Lysates were cleared by centrifugation at 15,000 × g for 10 min. Aliquots of total cell lysate were subjected to immunoblotting with antibodies as indicated in Figs. 1 and 5–7. Mouse primary antibody was used at the following dilution in each case: 1/1000 for either immunoprecipitation using antibodies as indicated in Figs. 6 and 7 and protein A- and G-Sepharose (Amersham Biosciences) or GST fusion protein interaction studies by incubation with the indicated GST fusion protein. This treatment was followed by SDS-PAGE and transfer
Identification and Characterization of SMPP-1M Kinase

RESULTS

Immunoblotting Studies—A 32-kDa SMPP-1M-associated kinase was detected previously by immunoblotting of rabbit bladder lysates with the anti-hZIPK antibody (16). We have used this antibody in immunoblotting studies of proteins expressed by native vascular smooth muscle cells and in studies of HEK293 cells expressing the cDNA for hZIPK (Fig. 1). In both VSMC cells and in heterologous expression studies of HEK293 cells the most prominent protein band detected by the anti-zIPK antibody was 52 kDa (Fig. 1, upper arrows). A 32-kDa band was also detected in both cell types (arrowhead) but was less prominent, especially in human VSMC lysates. The inclusion of protease inhibitors did not alter the amount of 32-kDa protein detected (+ lanes, Fig. 1). In the HEK293 cells expressing hZIPK, another protein of ~48 kDa was also prominent. Detection of this 48-kDa protein was also not affected by the inclusion of a mixture of protease inhibitors.

cDNA Library Screening for ZIPK and ZIP-like Kinase—A human aorta cDNA library containing 3.5 × 10^6 independent clones was screened for the presence of ZIPK and ZIP-like kinase by the colony hybridization technique. A 981-bp fragment of the 5’-end of hZIPK was used as the probe. This region of hZIPK cDNA was chosen for the probe design based on a previous report of homology between peptide fragments of ZIP-like kinase and the amino-terminal kinase domain of ZIPK (16) (Fig. 2A). The human aorta cDNA library was screened under both high and low stringency conditions. Using high stringency conditions, we obtained 18 positive clones. These positive clones were fully sequenced, and all 18 positive clones were identified as either full-length or fragments of hZIPK. Under low stringency conditions, 41 positive clones were obtained, and 31 were identified as full-length or fragments of hZIPK. We also cloned myosin light chain kinase (eight clones), the kinase domain of which has 48% amino acid sequence identity and 59% nucleotide sequence identity (25), as well as part of the ZIPK gene on chromosome 19 (two clones). ZIP-like kinase was not identified by either of the library screens.

PCR Studies to Search for ZIP-like Kinase and Detect Potential ZIPK Splice Variants—We designed degenerate PCR primers corresponding to the three peptide sequences derived originally from ZIP-like kinase with minor or uncertain sequence differences from hZIPK (Fig. 2B) (16). All DNA fragments amplified from the human aorta library using these degenerate primers were sequenced (Fig. 2C) but proved to be nonspecific, and no sequences corresponded to those of hZIPK or ZIP-like kinase.

To determine whether ZIP-like kinase is a splice variant of hZIPK, we searched for hZIPK splice variants in the human aorta cDNA library. hZIPK mRNA is derived from eight exons on chromosome 19. We designed PCR primers corresponding to each exon and two putative exons designated potential exons A and B, one 5’ to exon 1 (potential exon A) and the other between exon 2 and exon 3 (potential exon B) (Fig. 3A) based on predicted potential splice sites and variants (the NCBI LocusLink program). PCR was performed with 11 primer pairs designed to amplify both the known exons and potential exons A and B. Only the hZIPK exons were amplified. A number of DNA fragments were sequenced and proved to be of 100% identity to hZIPK (Fig. 3B). These results make the existence of a potential splice variant in human aorta of hZIPK unlikely.

Northern Blotting Studies—We investigated the expression of mRNA encoding ZIP-like kinase in various human tissues and cultured cells by performing Northern blot analyses. In all of these human tissues and cultured cells, a 5’-probe, based on hZIPK, was used at moderate stringency to detect hZIPK and any related transcripts. In all tissues and cells examined, a 2.3-kb band consistent with the predicted size of the hZIPK transcript was present, but no evidence of any other transcripts was noted (Fig. 4, A and B). ZIP-like kinase was co-purified with SMPP-1M originally from bladder smooth muscle (16). We therefore obtained human bladder tissue, isolated the mRNA, and performed separate Northern blot experiments (Fig. 4B, right panel). In human bladder, only a 2.3-kb band consistent with the predicted size of the hZIPK transcript was detected, and no evidence of any other transcript was noted. Re-probing of these blots with full-length hZIPK probe led to detection of the same bands and no others, indicating that these 2.3-kb bands are hZIPK mRNA (data not shown). We also investigated hZIPK and ZIP-like kinase expression in various mouse tissues, including brain, aorta, heart, lung, liver, spleen, kidney, uterus, and bladder. Among the mouse tissues, an ~2.3-kb band was detected. No other smaller mRNA species were detected (data not shown). We therefore could find no evidence that suggested the existence of an mRNA for a ZIP-like kinase in human and mouse tissues or in cultured cells.

Interaction of hZIPK with MBS—We investigated whether full-length hZIPK interacts with MBS. FLAG-tagged hZIPK was well expressed in HEK293 cells and distributed uniformly throughout the cytoplasm in these cells (Fig. 5, A and B). FLAG-hZIPK bound the C-terminal half of human MBS (GST-MBS with aa 681–1030). In contrast, neither GST-MBS with aa 847–1030 nor a mutant GST-MBS with aa 847–1030 in which the leucine zipper of MBS is disrupted by alanine substitution (designated GST-MBS 847–1030 L2M in Fig. 5) bound hZIPK. These data confirm an in vitro interaction between full-length hZIPK and the coiled coiled-containing domain of MBS (aa 681–847 of human MBS) (Fig. 5C). The binding of native ZIPK from human vascular smooth muscle cells to these GST-MBS fusion proteins was similarly observed (Fig. 5E). In reciprocal studies,
full-length ZIPK and N-terminal ZIPK both bound MBS, whereas GST-C-terminal ZIPK did not, supporting the idea that the amino-terminal half of ZIPK binds to MBS (Fig. 6D). Next, MBS and hZIPK were each immunoprecipitated from cells to test for an interaction between the two proteins in vivo. When MBS was immunoprecipitated from HEK293 cells, FLAG-hZIPK was present in the immunopellet (Fig. 6A), and, reciprocally, the immunoprecipitation of FLAG-hZIPK led to the recovery of MBS in the immunopellet (Fig. 6B). These data indicate that hZIPK and MBS are complexed in the cell. Finally, we studied whether the small GTPase RhoA might regulate the interaction we observed between hZIPK and MBS. In GST pull-down assay studies, no differences were detected in the level of ZIP kinase bound by MBS from native Rho with
either RhoN19 (dominant negative) or RhoQL (constitutively active) (data not shown). MLC phosphorylation by ZIP kinase also was assayed by isolating ZIP kinase from cells expressing wild type Rho, RhoN19, or RhoQL, but differences in the level of MLC phosphorylation were not detected in this assay (data not shown). When hZIPK was immunoprecipitated, however, co-immunoprecipitation of MBS was significantly diminished in the presence of a dominant negative mutant of RhoA, RhoN19 (Fig. 7). The constitutively active Rho protein, RhoQL, did not increase the level of ZIPK bound to MBS above the level seen with native (wild type) Rho in these studies (Fig. 7). These immunoprecipitation data support the belief that active Rho promotes the hZIPK-MBS interaction characterized in these studies.

**DISCUSSION**

Because the regulatory pathways that modulate SMPP-1M activity remain incompletely understood, we undertook this study to identify the endogenous SMPP-1M-associated kinase(s). A 32-kDa ZIP-like kinase had been found previously to co-purify with and inhibit SMPP-1M activity, whereas 52-kDa hZIPK had been shown to phosphorylate MLC and cause cell contraction (16, 22, 25). We set out to clone the SMPP-1M-associated ZIP-like kinase from vascular tissue, employing two methods to screen a highly representative human aorta cDNA library for evidence of ZIP-like kinase. In the first approach, we performed colony hybridization using a probe against the 5’-half of hZIPK, which, because of high homology, would be expected to identify both ZIP-like kinase and hZIPK. We also used a reverse transcription PCR approach with this human aorta library as the template and both degenerate primers and primers designed to identify hZIPK splice variants. In our studies, only hZIPK but not any ZIP-like kinase was present in this library.

As alternative approaches, we also used both the 5’-catalytic domain and full-length hZIPK as probes in both vascular and non-vascular cellular RNA Northern hybridization experiments. A variety of human tissues were hybridized with the radiolabeled 5’-end of hZIPK and exposed to a PhosphorImager (Amersham Biosciences). Northern filters prepared from human primary vascular cell lines and human bladder tissue RNA, Polya(+) RNAs (2 μg) from various cultured cells and human bladder were loaded in each lane. The filters were hybridized with the radiolabeled 5’-end of hZIPK as a probe and exposed to a PhosphorImager. AoSMC, primary human aortic smooth muscle cell; CoSMC, human coronary arterial smooth muscle cell; PuSMC, human pulmonary arterial smooth muscle cell; RaSMC, human radial artery smooth muscle cell; HUVEC, human umbilical vein endothelial cells.

**FIG. 3.** Investigation of potential mRNA splice variants of human ZIP kinase. A, structure of the hZIPK gene on chromosome 19. The two predicted exons are labeled A and B. Exons 1, 2, 3, 4, 5, 6, 7, and 8 each encode 1–62, 63–423, 424–553, 554–603, 604–629, 630–783, 784–829, and 830–1365 bp of ZIP kinase, respectively. Each PCR primer was designed to correspond to each exon and predicted exon using the NCBI LocusLink program. Arrows indicate the forward and reverse primers corresponding to known exons; arrowheads indicate the forward primer corresponding to predicted potential exons A and B. B, PCR products were size-fractionated by agarose gel electrophoresis. A large number of DNA fragments amplified with this approach were sequenced; all sequences proved to be identical to hZIPK. M, 1-kb DNA ladder marker.

**FIG. 4.** Northern blotting studies of hZIPK mRNA expression in non-vascular and vascular tissues. A, multi-tissue Northern filters (Clontech). These filters, along with 1.5 μg of poly(A)+ RNA from various human tissues, were hybridized with the radiolabeled 5’-end of hZIPK and exposed to a PhosphorImager (Amersham Biosciences). B, Northern filters prepared from human primary vascular cell lines and human bladder tissue RNA. Polya(+) RNAs (2 μg) from various cultured cells and human bladder were loaded in each lane. The filters were hybridized with the radiolabeled 5’-end of hZIPK as a probe and exposed to a PhosphorImager. AoSMC, primary human aortic smooth muscle cell; CoSMC, human coronary arterial smooth muscle cell; PuSMC, human pulmonary arterial smooth muscle cell; RaSMC, human radial artery smooth muscle cell; HUVEC, human umbilical vein endothelial cells.
identification and Characterization of SMPP-1M Kinase

**Fig. 5. Interaction of ZIPK with MBS in vitro.** A. HEK293 cells were transfected with hZIPK plasmid or control plasmid, and cell lysates were immunoblotted (IB) with either anti-ZIPK (top) or anti-FLAG (bottom) antibodies. The arrow and arrowheads indicate endogenous hZIPK and FLAG-tagged hZIPK, respectively. B. HEK293 cells transiently transfected with hZIPK were fixed and stained with anti-FLAG antibody and visualized with immunofluorescence microscopy. Note that ZIP kinase is distributed uniformly throughout the cytoplasm in these cells. C. HEK293 cells transfected with hZIPK were lysed, and the lysates were then used in GST pull-down protein-protein interaction studies. Binding of ZIPK was probed using anti-FLAG antibody. The lane marked GST represents GST alone. GST-MBS constructs expressing either aa 681–1030 or aa 847–1030 are represented by the lanes marked GST-MBS 681–1030 or GST-MBS 847–1030. The lane marked GST-MBS 847–1030 LZM represents the construct expressing the domain of MBS with the leucine residues in the leucine zipper of the domain mutated to alanines. D. HEK293 cells transfected with hZIPK were lysed, and the lysates were then used in GST pull-down protein-protein interaction studies. Binding of ZIPK was probed using the anti-ZIPK antibody. The lane marked GST represents GST alone. GST-MBS constructs expressing either aa 681–1030 or aa 847–1030 are represented by the lanes marked GST-MBS 681–1030 or GST-MBS 847–1030. The lane marked GST-MBS 847–1030 LZM represents the construct expressing the domain of MBS with the leucine residues in the leucine zipper of the domain mutated to alanines (14). The far right lane shows a recombinant ZIPK (rZIPK) positive control.

**Fig. 6. Interaction of ZIPK with MBS in cells.** HEK293 cells transfected with hZIPK were lysed, and lysates were then used in immunoprecipitation (IP) studies of human MBS (panel A) or ZIPK (panel B), followed by immunoblotting (IB) of ZIPK or MBS, respectively.

We also tested for the binding of hZIPK to MBS in several ways. Heterologous expression of hZIPK demonstrated that hZIPK binds directly to the coiled coil-containing domain of MBS in GST pull-down protein-protein interaction studies and to complexes with MBS in co-immunoprecipitation experiments, strongly supporting the idea that hZIPK interacts with MBS in the cell. Furthermore, disruption of RhoA activation by overexpression of a dominant negative RhoA mutant inhibited this hZIPK-MBS interaction in cells. We did not detect an effect of RhoN19 on hZIPK-MBS binding using GST fusion proteins or in a MLC phosphorylation assay using immunoprecipitated hZIPK derived from cells expressing wild type or mutant Rho protein. These negative data support either the reduced sensitivity of these assays compared with co-immunoprecipitation or the need for additional cellular proteins for Rho to regulate the hZIPK-MBS interaction. Taken together, the data support the hypothesis that the interaction between hZIPK and MBS may be regulated by Rho and that hZIPK may play a role in PP1M regulation by RhoA.

Studies in both animals and humans have shown that SMPP-1M activity is regulated in health and in disease. Hyperactivity of the RhoA pathway leading to SMPP-1M inhibition and blood vessel contraction has been shown in hypertensive states, and enzyme inhibitors that prevent SMPP-1M inhibition are promising new treatments for cardiovascular disorders (26, 27). Our data establish that hZIPK is the SMPP-1M-associated kinase and support the hypothesis that the hZIPK-SMPP-1M interaction is regulated by RhoA. Future studies will be directed at defining the role of hZIPK in the modulation of SMPP-1M activity by vasoconstrictor agonists and other potential mechanisms through which hZIPK regulates vascular smooth muscle cell function.

**REFERENCES**

1. Hartshorne, D. J. (1987) in Physiology of the Gastrointestinal Tract (Johnson, D. R., ed.) pp. 428–482. Raven Press, Ltd., New York.
2. Somlyo, A. P., and Somlyo, A. V. (1994) Nature 372, 231–236.
3. Alessi, D., MacDougall, L. K., Sola, M. M., Ibebe, M., and Cohen, P. (1992) Eur. J. Biochem. 210, 1023–1035.
4. 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.
5. Khatri, J. J., Joyce, K. M., Bronzovich, F. V., and Fisher, S. A. (2001) J. Biol. Chem. 276, 37250–37257.
6. Etter, E. F., Tio, M., Wardle, R. L., Brautigan, D. L., and Murphy, R. A. (2001) J. Biol. Chem. 276, 34681–34685.
7. Lee, M. R., Li, L., and Kitazawa, T. (1997) J. Biol. Chem. 272, 5063–5068.
8. Wu, X., Somlyo, A. V., and Somlyo, A. P. (1996) Biochem. Biophys. Res. Com. 220, 658–663.
9. Kamm, K. E., and Stull, J. T. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 593–620.
10. Taylor, D. A., and Stull, J. T. (1988) J. Biol. Chem. 263, 14456–14462.

\[ \text{REFERENCES} \]

1. Hartshorne, D. J. (1987) in Physiology of the Gastrointestinal Tract (Johnson, D. R., ed.) pp. 428–482. Raven Press, Ltd., New York.
2. Somlyo, A. P., and Somlyo, A. V. (1994) Nature 372, 231–236.
3. Alessi, D., MacDougall, L. K., Sola, M. M., Ibebe, M., and Cohen, P. (1992) Eur. J. Biochem. 210, 1023–1035.
4. 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.
5. Khatri, J. J., Joyce, K. M., Bronzovich, F. V., and Fisher, S. A. (2001) J. Biol. Chem. 276, 37250–37257.
6. Etter, E. F., Tio, M., Wardle, R. L., Brautigan, D. L., and Murphy, R. A. (2001) J. Biol. Chem. 276, 34681–34685.
7. Lee, M. R., Li, L., and Kitazawa, T. (1997) J. Biol. Chem. 272, 5063–5068.
8. Wu, X., Somlyo, A. V., and Somlyo, A. P. (1996) Biochem. Biophys. Res. Com. 220, 658–663.
9. Kamm, K. E., and Stull, J. T. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 593–620.
10. Taylor, D. A., and Stull, J. T. (1988) J. Biol. Chem. 263, 14456–14462.
11. Eto, M., Senba, S., Morita, F., and Yazawa, M. (1997) FEBS Lett. 410, 356–360
12. Eto, M., Kitazawa, T., Yazawa, M., Mukai, H., Ono, Y., and Brautigan, D. L. (2001) J. Biol. Chem. 276, 29072–29078
13. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, R., Feng, J., Nakano, T., Okawa, K., Iwamata, A., and Kaibuchi, K. (1996) Science 273, 245–248
14. Surks, H. K., Richards, C. T., and Mendelsohn, M. E. (2003) J. Biol. Chem. 278, 51484–51493
15. Trinkle-Mulcahy, L., Ichikawa, K., Hartshorne, D. J., Siegman, M. J., and Butler, T. M. (1995) J. Biol. Chem. 270, 18191–18194
16. MacDonald, J. A., Borman, M. A., Muranyi, A., Somlyo, A. V., Hartshorne, D. J., and Haystead, T. A. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2419–2424
17. Muranyi, A., MacDonald, J. A., Deng, J. T., Wilson, D. P., Haystead, T. A., Walsh, M. P., Erdodi, F., Kiss, E., Wu, Y., and Hartshorne, D. J. (2002) Biochem. J. 366, 211–216
18. Muranyi, A., Zhang, R., Liu, F., Hirano, K., Ito, M., Epstein, H. F., and Hartshorne, D. J. (2001) FEBS Lett. 493, 80–84
19. Broustas, C. G., Grammatikakis, N., Eto, M., Dent, P., Brautigan, D. L., and Kasid, U. (2002) J. Biol. Chem. 277, 3053–3059
20. Borman, M. A., MacDonald, J. A., Muranyi, A., Hartshorne, D. J., and Haystead, T. A. (2002) J. Biol. Chem. 277, 23441–23446
21. Kawai, T., Matsumoto, M., Takeda, K., Sanjo, H., and Akira, S. (1998) Mol. Cell. Biol. 18, 1642–1651
22. Niire, N., and Ikebe, M. (2001) J. Biol. Chem. 276, 29567–29574
23. Ichiba, T., Hashimoto, Y., Nakayama, K., Kuratsu, Y., Tanaka, S., Kurata, T., Mochizuki, N., and Matsuda, M. (1999) J. Biol. Chem. 274, 14376–14381
24. Grunstein, M., and Hogness, D. S. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3961–3965
25. Murata-Hori, M., Suizu, F., Iwasaki, T., Kikuchi, A., and Hossya, H. (1998) FEBS Lett. 451, 81–84
26. Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997) Nature 389, 990–994
27. Kandabashi, T., Shimokawa, H., Mukai, Y., Matoba, T., Kunihiro, I., Morikawa, K., Ito, M., Takahashi, S., Kaibuchi, K., and Takeshita, A. (2002) Arterioscler. Thromb. Vasc. Biol. 22, 243–248
Identification and Characterization of Zipper-interacting Protein Kinase as the Unique Vascular Smooth Muscle Myosin Phosphatase-associated Kinase
Akira Endo, Howard K. Surks, Seibu Mochizuki, Naoki Mochizuki and Michael E. Mendelsohn

J. Biol. Chem. 2004, 279:42055-42061.
doi: 10.1074/jbc.M403676200 originally published online July 30, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403676200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 17 of which can be accessed free at http://www.jbc.org/content/279/40/42055.full.html#ref-list-1