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Tissue expression and subcellular localization of Mipu1, a novel myocardial ischemia-related gene

G. Wang^{1,3*}, X. Zuo^{2*}, L. Jiang¹, K. Wang¹, X. Wei¹, B. Zhang¹ and X. Xiao¹

¹Laboratory of Shock, Department of Pathophysiology, ²Department of Rheumatology and Clinical Immunology, Xiangya School of Medicine, Central South University, Changsha, Hunan, China

³Department of Digestive Internal Medicine, People's Hospital of Pingxiang City, Pingxiang, Jiangxi, China

Correspondence to: X. Xiao, Department of Pathophysiology, Xiangya School of Medicine, Central South University, 110 Xiangya Road, Changsha, Hunan 410008, China

Fax: +86-731-235-5019. E-mail: xianzhongxiao@xysm.net

Myocardial ischemic preconditioning up-regulated protein 1 (Mipu1), a novel zinc finger protein, was originally cloned using bioinformatic analysis and 5' RACE technology of rat heart after a transient myocardial ischemia/reperfusion procedure in our laboratory. In order to investigate the functions of Mipu1, the recombinant prokaryotic expression vector pQE31-Mipu1 was constructed and transformed into *Escherichia coli* M15(pREP4), and Mipu1-6His fusion protein was expressed and purified. The identity of the purified protein was confirmed by mass spectrometry. The molecular mass of the Mipu1 protein was 70.03779 kDa. The fusion protein was intracutaneously injected to immunize New Zealand rabbits to produce a polyclonal antibody. The antibody titer was approximately 1:16,000. The antibody was tested by Western blotting for specificity and sensitivity. Using the antibody, it was found that Mipu1 was highly expressed in the heart and brain of rats and was localized in the nucleus of H9c2 myogenic cells. The present study lays the foundation for further study of the biological functions of Mipu1.

Key words: Mipu1; Purification; Expression; Polyclonal antibody

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*These authors contributed equally to this study.

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Introduction

During myocardial ischemia or reperfusion, many genes such as c-fos, c-jun, junB, Egr-1, and HSP70 can be up-regulated (1-3), and some of these myocardial genes have been considered to be involved in the endogenous cardioprotection against myocardial ischemia-reperfusion injury. In our laboratory, Yuan and colleagues (4) found that myocardial ischemic preconditioning up-regulated protein 1 (Mipu1) was up-regulated in rat heart after a transient myocardial ischemia-reperfusion procedure, and they cloned the full length cDNA of the Mipu1 gene (GenBank

accession No. AY221750). Jiang et al. (5) reported that Mipu1 was a nuclear and DNA binding protein that bound a specific DNA sequence, TGTCTTATCGAA, with TCTTA as the core sequence. This suggested that Mipu1 might function as a transcription factor. Since Mipu1 is a novel gene, its function and expression profile is still to be clarified. In the present study, the cDNA gene encoding rat Mipu1 was expressed in *Escherichia coli* and the Mipu1-6His fusion protein was purified and injected into New Zealand rabbits for the production of an anti-Mipu1 polyclonal antibody. The antibody was used to detect the abundance of Mipu1 in various rat tissues and the expres-

sion and subcellular localization of Mipu1 in H9c2 myogenic cells.

Material and Methods

Materials, strains, enzymes, and kits

The plasmid pcDNA3.1-Mipu1 was constructed in our previous study (4). The pQE31 vector was purchased from Qiagen (Germany). Strain *E. coli* M15(pREP4) was preserved in our laboratory. Restriction endonucleases *Bam*HI, *Kpn*I, and T4 DNA ligase were purchased from Promega (USA). Pfu DNA polymerase was purchased from TaKaRa (Japan). The gel extraction kit and plasmid extraction kits were purchased from Qiagen. DEAE-cellulose (DE52) was obtained from Whatman (USA). Pre-packed DEAE Sepharose-fast flow and Q Sepharose-fast flow columns designed for use with syringes were from GE Health Care (USA). The Ab buffer kit was purchased from Amersham Pharmacia Biotech (Sweden).

Construction of pQE31-Mipu1 prokaryotic expression vector

Gene-specific primers were designed according to the sequence of rat Mipu1. The forward primer (5'-AAAAGG ATCCAATGCCTGCAGCCCGAGGG-3') containing a *Bam*HI site and the reverse primer (5'-GGGGTACCATTA GGACATTTCTCCGAATGTATAC-3') containing a *Kpn*I site were used. The complete open reading frame of Mipu1 was amplified by the polymerase chain reaction (PCR) from pcDNA3.1-Mipu1 by using a Bio-Rad (USA) DNA Engine Pelter thermal cycler under the following conditions: 95°C for 3 min, followed by 35 cycles of amplification (95°C, 30 s; 56°C, 30 s; 72°C, 1.5 min). The resulting product was ligated to pQE31 after digestion with *Bam*HI and *Kpn*I, and then transformed into strain *E. coli* M15 (pREP4). The ligated clones were screened by PCR using the primers cited above and by *Bam*HI and *Kpn*I double digestion. Potential clones were confirmed by DNA sequencing (Invitrogen, China).

Expression, purification and identification of Mipu1-6His fusion protein

Expression of Mipu1-6His fusion protein was monitored after the recombinant plasmid pQE31-Mipu1 was transformed into *E. coli* strain M15(pREP4) and selected on an agar A plate (Bio Basic Inc., Canada) containing 100 µg/mL ampicillin (Sigma, USA). The transformants were inoculated into 3 mL Luria-Bertani liquid medium containing ampicillin (100 µg/mL), incubated overnight at 37°C with constant shaking, and then transferred to 1 L of fresh medium and incubated for another 4 h until the absorbance

(A600) of the cultured cells reached 0.6. Expression of the Mipu1-6His fusion protein was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma). To optimize the induction conditions, different cell concentrations (A600: 0.3, 0.6, 1.0, and 1.5), different concentrations of IPTG (0.2, 0.4, 0.6, 0.8, and 1.0 mM) inducing the concentrations of the cells to an A600 of 0.6, and different induction times (0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, and 8.0 h) inducing the concentrations of the cells to an A600 of 0.6 were tested. As a result, the Mipu1-6His fusion protein was induced to express a maximize yield using 1 mM IPTG for 4 h at an A600 of 0.6. After induction, cells were lysed in 2X sample buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 0.1 M DTT) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide gel). The noninduced control culture was analyzed in parallel.

To purify the Mipu1-6His fusion protein, the induced bacterial cell pellet was resuspended in 40 mL of ice-cold binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9). The cell suspension was incubated with lysozyme (0.2 µg/µL) and sonicated on ice for 5-10 min until the sample was clear. The lysate was centrifuged at 14,000 *g* for 30 min at 4°C. The supernatant was filtered using a 0.45-µm pore size membrane and applied to a His-bind resin column (Novagen, USA), which was previously equilibrated with three column volumes of sterile deionized water, five column volumes of charge buffer (50 mM NiSO₄) and three column volumes of binding buffer, in that order. The column was then washed with ten column volumes of binding buffer and six column volumes of wash buffer with 100 mM imidazole. During batch purification, most of the contaminant *E. coli* proteins were eluted with 100 mM imidazole, whereas the target proteins were eluted with a gradient of 200-1000 mM imidazole. Ion exchange resin with an equal volume of Pellicon-enriched (~10-fold) cell-free flask shaken culture supernatant was used for small-scale trials. Gravity-flow columns with DE52 resin (1 mL, disposable) and pre-packed DEAE Sepharose™ and Q Sepharose™ Fast Flow (1 mL, reusable) columns were tested for further purification of the Mipu1-6His fusion protein. Initial experiments were conducted for selection of the optimal binding buffer, subsequent wash steps and elution of the target. The equilibration buffers tested included 50 mM Tris-HCl, pH 7.0, 7.5, 8.0, 8.5, and 8.8, respectively. For the wash, 50 mM sodium acetate, pH 6.8, sodium acetate combined with MES (MES-acetate, pH 6.8) were used, and for final elution, 50 mM sodium acetate, pH 5.0, and MES-acetate, pH 5.0, were used. Additionally, a stepwise salt gradient was also performed to compare and evaluate selective elution of the target using

100-500 mM NaCl. The fractionated samples were analyzed by monitoring A280 and representative eluates after nickel affinity and ion exchange columns. The products were analyzed by SDS-PAGE and Coomassie blue staining.

To further identify the Mipu1-6His fusion protein, the recombinant protein was separated by SDS-PAGE and then confirmed by Western blotting according to a standard protocol using the anti-His monoclonal antibody (Qiagen). The proteins on the polyacrylamide gel were transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membrane using a trans-blot SD semi-dry electrophoretic transfer cell (Bio-Rad), and the membrane was then incubated with the anti-6His monoclonal antibody (TIANGEN, Beijing, China) at a dilution of 1:1000 for 2 h at room temperature (RT) after incubation for 4 h with blocking buffer (2% BSA in Tris-buffered saline, TBS) at RT. The membrane was washed three times with TBS containing 0.1% Tween 20 (TBST). The immunoreactive protein bands were detected with peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, USA).

Mass spectrometry analysis

The protein band corresponding to Mipu1-6His fusion protein was excised from the SDS-polyacrylamide gel, minced into small pieces, and destained twice for 15 min each in 200 μ L of 25 mM ammonium bicarbonate and 50% acetonitrile. The wet gel pieces were dehydrated in acetonitrile for 10 min and dried in a vacuum centrifuge. This step was followed by reduction in 2 mM DTT for 1 h and alkylation in 20 mM iodoacetamide for 30 min prior to digestion with trypsin (Promega) at a ratio of 1:20 (w/w) for 4 h at 37°C. The resulting peptides were extracted in 50 μ L 50% acetonitrile, 5% formic acid for 20 min, and then 75% acetonitrile and 5% formic acid for 10 min. The solutions from the two extraction steps were combined and dried in a vacuum centrifuge and the peptides were rehydrated in 10 μ L 0.1% formic acid. The peptide sample was first separated on an Ettan MDLC system (GE Healthcare) and then subjected to mass spectrometry (MS) analysis on a Finnigan-LTQ Mass Spectrometer (Thermo Electron, USA). A gradient consisting of 0-60% solvent B for 60 min and 60-100% solvent B for 5 min (solvent A, 0.1% formic acid in water; solvent B, 84% acetonitrile, 0.1% formic acid in water) at a flow rate of 200 nL/min was used to elute peptides from the reverse phase column and transfer them to the MS through an integrated electrospray emitter tip. For every full scan on the LTQ mass spectrometer, the five most abundant peaks were subjected to MS/MS analysis. Bio Works 3.2 (Finnigan Xcalibur) was used to analyze all spectra from MS and MS/MS and determine peptide sequences. Finally, a search was performed for protein iden-

tification using the rat database provided in the software. Modification was permitted to allow for the detection of carboxyamidomethylated cysteine.

Immunization of New Zealand rabbits using purified Mipu1-6His fusion protein

All protocols involving experimental animals followed the local institutional guidelines for animal care, which are comparable to the Guide for the Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research (National Institutes of Health publication No. 85-23, revised 1996). Three young female New Zealand rabbits were selected and 300 μ g purified Mipu1-6His fusion protein in Freund's complete adjuvant was injected into each rabbit. To reinforce immunity, 150 μ g Mipu1-6His purified fusion protein was injected into the rabbits on the 4th, 7th, 9th, and 11th week after the first injection. At various stages, a blood drop was drawn and centrifuged to prepare antisera for titer analysis.

Determination of the anti-Mipu1 polyclonal antisera titer by ELISA

Antiserum titer was measured using an indirect enzyme-linked immunosorbent analysis (ELISA). The purified antigen (purified Mipu1-6His fusion protein), diluted to 10 μ g/mL in 50 mM carbonate salt buffer, pH 9.6, was coated on plates at 100 μ L aliquot per well at 4°C overnight. The wells were washed three times with phosphate-buffered saline (PBS)-Tween buffer (0.05% Tween 20 in PBS). The coated wells were blocked with 200 μ L 3% BSA for 1 h at 37°C and then incubated with 150 μ L polyclonal antibodies against Mipu1 at different dilutions (from 1:1000 to 1:25,600). After incubation for 2 h at 37°C, the wells were incubated with 150 μ L horse radish peroxidase-conjugated goat anti-rabbit IgG (dilution 1:5000, Sigma) for 1 h at 37°C after thorough washing. Peroxidase activity on the immunoplate was detected using O-phenylenediamine and H₂O₂ as enzyme substrates. Color development was stopped with 2 M of H₂SO₄ and absorbance was measured at 490 nm using a Microplate Reader (Bio-Rad). Antibody dilutions (1000- to 256,000-fold) were reacted with an equal amount (1 μ g) of the fusion protein. The antiserum titer was defined as the highest dilution of serum at which the A490 ratio (A490 of post-immunization serum/A490 of preimmunization serum) was greater than 2.1. During the 12th week after the first injection when the antiserum titer was 1:16,000, the rabbits were bled and the antiserum was collected.

Purification of anti-Mipu1 antiserum

The Ab buffer kit was used to purify the anti-Mipu1

antiserum. Collection tubes were prepared by adding 60 to 200 μ L of neutralizing buffer per mL of fraction to be collected. In this way the final pH of the eluted antibodies was approximately neutral, thus preserving the activity of acid-labile IgG. Ab SpinTrap columns were placed in a 2-mL microcentrifuge tube to collect liquid. The Ab SpinTrap column was inverted and shaken repeatedly to resuspend the medium and the column was equilibrated by adding 600 μ L binding buffer. Anti-Mipu1 antiserum was added to the column and incubated for 4 min with gentle mixing. The microcentrifuge tube was emptied and washed with 600 μ L binding buffer and then centrifuged for 30 s at 70–100 *g*. The bound antibody was eluted by adding 400 μ L elution buffer to the column. The column was placed in a 2-mL microcentrifuge tube containing 30 μ L neutralizing buffer and centrifuged for 30 s at 7000 *g*. This step was repeated and the second elution in a fresh 2-mL microcentrifuge tube containing 30 μ L neutralizing buffer was collected. The eluted fraction contained the purified antibody. As determined by the Bradford assay (Bio-Rad), the concentration of the antibody was 11.64 mg/mL. To determine whether the polyclonal antibody could bind specifically to Mipu1, the polyclonal antibody was identified by Western blotting using the Mipu1-6His fusion protein.

Cell culture and transfection

H9c2 myogenic cells were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal calf serum (Gibco), 2 mM/L-glutamine, 20 U/mL penicillin, and 20 mg/mL streptomycin. The culture was kept in an incubator with 5% CO₂ and 95% humidified air at 37°C. Cells were seeded onto a 6-well culture plate and grown to 50–70% confluence. Cells were washed with PBS, and 1.5 mL serum-free medium was added to each well. PcDNA3.1 or pcDNA3.1-Mipu1 with full-length Mipu1 cDNA was transfected transiently into H9c2 myogenic cells using Lipofectamine 2000 (Invitrogen) for 24 h according to manufacturer instructions.

Identification of Mipu1 in H9c2 myogenic cells by Western blotting

Cells were washed with 1X PBS and resuspended with 5 volumes of cold lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% Nonidet P-40) supplemented with a protease inhibitor mixture (Roche Applied Science, Switzerland). The cell lysate was incubated on ice for 30 min and then centrifuged for 10 min at 4°C. The protein content of the supernatant was determined by the Bradford assay (Bio-Rad). After equal amounts of protein (20 μ g) were loaded onto each lane and separated by SDS-PAGE, the gel was immersed in the transfer

buffer (48 mM Tris-HCl, 39 mM glycine, and 20% methanol, pH 9.2) and the proteins were transferred to a PVDF membrane. The membrane was incubated for 4 h with blocking buffer (2% BSA in TBS) at RT. After being washed three times (10 min each) with TBST buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween), the membrane was incubated with the anti-Mipu1 polyclonal antibody (1:200) and anti-Myc-Tag (1:1000) monoclonal antibody (cell signaling) for 2 h at RT, and then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Sigma) or horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000, Sigma) at RT after thorough washing. The immunoreactive bands were visualized using DAB (Boster Biological Technology, China). Anti-GAPDH antibody (1:5000, Sigma) was used to normalize for equal amounts of proteins.

Determination of the sub-cellular localization of the rat Mipu1 protein

H9c2 myogenic cells were cultured on glass coverslips in a 6-well plate. After washing with cold PBS, cells were fixed with 2 mL 4% paraformaldehyde for 20 min. Cells were then washed three times with cold PBS and permeabilized with 2 mL PBS with 0.1% Triton X-100 for 15 min. After washing four times with cold PBS, cells were incubated with Mipu1 polyclonal antibody (diluted 1:50 with 2% BSA) for 2 h at 37°C. Cells were washed three times with PBS and then incubated with the secondary antibody, CY3-conjugated goat anti-rabbit IgG (Santa Cruz) for 1 h at 37°C. To detect the cell nucleus, specimens were further incubated with Hoechst 33258 (10 μ g/mL; Sigma) for 20 min. Background staining was removed by washing three times with PBS. The washed sections were mounted in anti-fade solution and photographed by laser scanning confocal microscopy (Nikon, USA). Images were processed with Adobe Photoshop, version 7.0 (Adobe Systems, USA).

Statistical analysis

Data are reported as mean \pm SEM of the indicated number of separate experiments. Statistical comparison was performed using the unpaired two-tailed Student *t*-test, with the level of significance set at $P < 0.05$.

Results

Purification of the recombinant Mipu1-6His fusion protein

Mipu1-6His fusion protein was first purified using the His-bind purification kit (Novagen) as described in Methods. We used 10% SDS-PAGE with Coomassie blue staining to examine the solubility of the Mipu1-6His fusion

protein and the optimal concentration of imidazole for elution. The Mipu1-6His fusion protein was induced with IPTG (cell number: 2×10^{12}) in soluble form (Figure 1A, lane 1), and the expression level of Mipu1-6His fusion protein without the induction of IPTG (cell number: 2×10^{12}) was much less (Figure 1A, lane 2). During batch purification, most of the contaminant *E. coli* proteins were eluted with 100 mM imidazole, whereas the target proteins were eluted with a gradient of 200-1000 mM imidazole (Figure 1A, lanes 3-5). At these points, the Mipu1-6His fusion protein was at least 85% purity. The Mipu1-6His fusion protein was at least 98% purity after ion-exchange chromatography (Figure 1B, lane 1). The identification of the Mipu1-6His fusion protein was further confirmed by Western blotting using the anti-His monoclonal antibody. An expected 70-kDa band of Mipu1-6His fusion protein was detected (Figure 1C, lane 1).

Mass spectrometry of recombinant Mipu1-6His protein

To determine the exact molecular weight and to further confirm the identity of the protein, purified Mipu1-6His fusion protein was subjected to MS analysis (Figure 2). The sequences of peptides derived from trypsin digestion were determined and searched against the rat database. The result showed that the peptides correspond to Mipu1 protein with 608 amino acid residues (AY221750, http://www.expasy.ch/cgi-bin/pi_tool) and a molecular mass of 70.03779 kDa (Table 1). The sequencing of 13 peptides accounting for 172 amino acid residues of the 608 residues present in Mipu1 corresponds to 28% coverage of the molecule by MS.

Titer and specificity analysis of anti-Mipu1 polyclonal antibody

Antiserum (about 20 μ g) was run on SDS-PAGE and stained with Coomassie blue. The heavy chain and light chain of the polyclonal antibody were shown but there were some nonspecific band (Figure 3A, lane 1). After purification with the Ab buffer kit, the heavy chain and light chain of the polyclonal antibody were clearly visible (Figure 3B, lane 1). The polyclonal antibody was identified by Western blotting using the Mipu1-6His fusion protein, which showed that anti-Mipu1 polyclonal antibody could specifically identify the Mipu1-6His fusion protein (Figure 3C, lane 1).

Expression of Mipu1 in H9c2 myogenic cells

Anti-Mipu1 polyclonal antibody was used to test for the presence of Mipu1 in the lysate of H9c2 myogenic cells and the H9c2 myogenic cells transfected with pcDNA3.1-Mipu1 (Figure 4A; $P < 0.05$). Anti-Myc-Tag monoclonal

antibody (at a dilution of 1:1000) was used to test the presence of the exogenous Mipu1-Myc fusion protein from the expression of pcDNA3.1-Mipu1 plasmid because there was a Myc-tag on the pcDNA3.1-Mipu1 plasmid (Figure 4B; $P < 0.05$). The 70-kDa bands of Mipu1 protein were

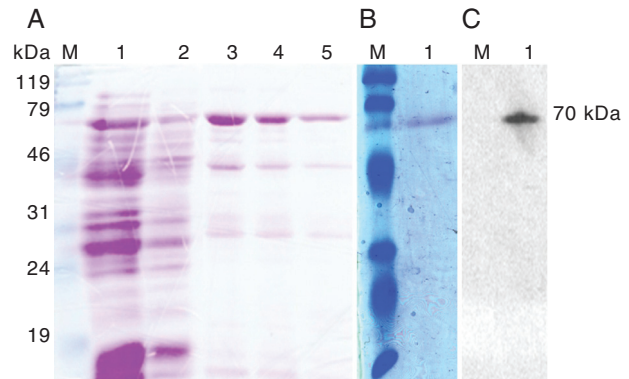


Figure 1. Expression, purification and identification of Mipu1-6His fusion protein. A, Purification of Mipu1-6His fusion protein using the His-bind purification kit, after 10% SDS-PAGE with Coomassie blue staining. M, Protein molecular weight markers; lane 1, whole lysate of *Escherichia coli* transformed with pQE31-Mipu1 and induced with IPTG; lane 2, whole lysate of *E. coli* transformed with pQE31-Mipu1 without induction with isopropyl- β -D-thiogalactopyranoside; lanes 3-5, protein eluted from the nickel affinity column with 200, 500, 1000 mM imidazole, respectively. B, Purification of Mipu1-6His fusion protein by ion-exchange chromatography after 10% SDS-PAGE with Coomassie blue staining. M, Protein molecular weight markers; lane 1, Mipu1-6His fusion protein. C, Identification of purified Mipu1-6His fusion protein by Western blotting using anti-His monoclonal antibody. M, Protein molecular weight markers; lane 1, purified Mipu1-6His fusion protein.

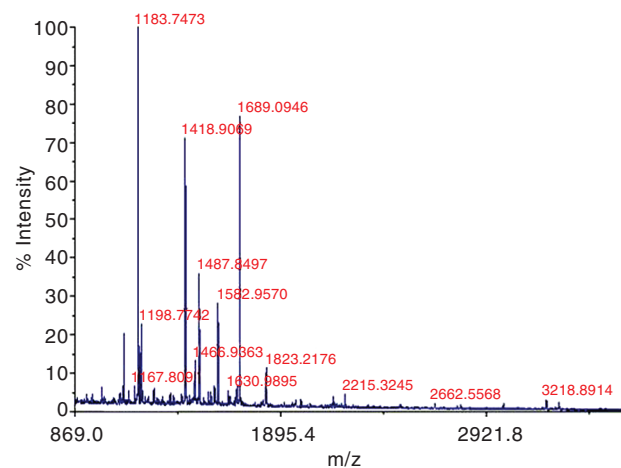


Figure 2. Mass spectra of peptides of Mipu1-6His fusion protein digested by trypsin.

Table 1. Database match of Mipu1 peptides.

Peptide	Sequence	Amino acids	Molecular weight
1	MPAARGKSKSK	1-11	1160.40
2	APVTFGDLAIYFSQEEWELSPNQK	12-36	2956.26
3	DLYEDVMLENYHNLVSVGLACR	37-58	2553.89
4	PNIIALLEK	60-68	1010.24
5	APWMVEPSR	71-80	1072.25
6	RGPELGSK	82-89	842.95
7	TSDSCPSLSPHHNNHAIDSIHQCR	233-256	2656.85
8	TYQSENPFPCR	321-331	1345.45
9	VHLNIHSEEK	430-439	1205.34
10	AFSQAHLAQHER	505-517	1481.59
11	PYECNECGK	552-560	1042.15
12	AFSSGSDLIR	561-570	1052.15
13	HQSIHSEEMS	599-608	1184.25

Peptides were identified in the SWISS-PROT annotated protein sequence database.

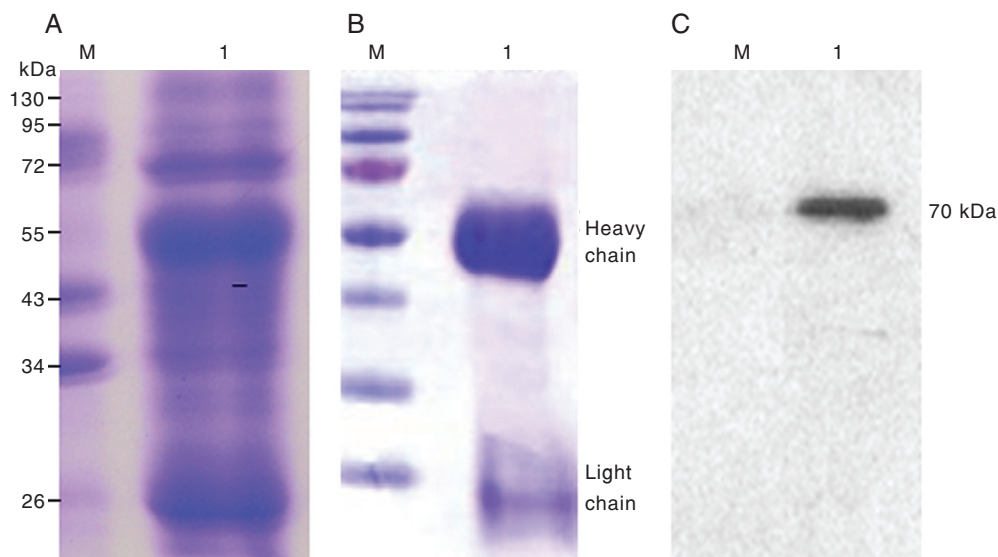


Figure 3. Identification of anti-Mipu1 polyclonal antibody. A, Anti-Mipu1 antiserum run on SDS-PAGE with Coomassie blue staining. M, Protein molecular weight markers; lane 1, anti-Mipu1 antiserum. B, Purified anti-Mipu1 polyclonal antibody run on SDS-PAGE with Coomassie blue staining. M, Protein molecular weight markers; lane 1, anti-Mipu1 polyclonal antibody. C, Specific binding of anti-Mipu1 polyclonal antibody to purified Mipu1-6His fusion protein as shown by Western blotting. M, Protein molecular weight markers; lane 1, combination of anti-Mipu1 polyclonal antibody and Mipu1-6His fusion protein.

detected as expected. The results confirmed that the anti-Mipu1 polyclonal antibody could detect endogenous Mipu1 protein expressed in H9c2 myogenic cells (Figure 4A,B, lanes 1) and exogenous pcDNA3.1-Mipu1 protein transfected into H9c2 myogenic cells (Figure 4A,B, lanes 2). This result indicated that H9c2 myogenic cells could express endogenous Mipu1 protein and exogenous Mipu1 protein from the plasmid pcDNA3.1-Mipu1.

Expression of Mipu1 in rat tissues

Western blotting showed that Mipu1 protein was expressed in a tissue-dependent manner (Figure 5). The highest levels of Mipu1 protein were detected in the heart and brain (vs heart, $P > 0.05$), moderate levels were detected in liver, kidney, lung, ovary, and testis (vs heart, $P < 0.05$), and very low levels in the spleen (vs heart, $P < 0.05$).

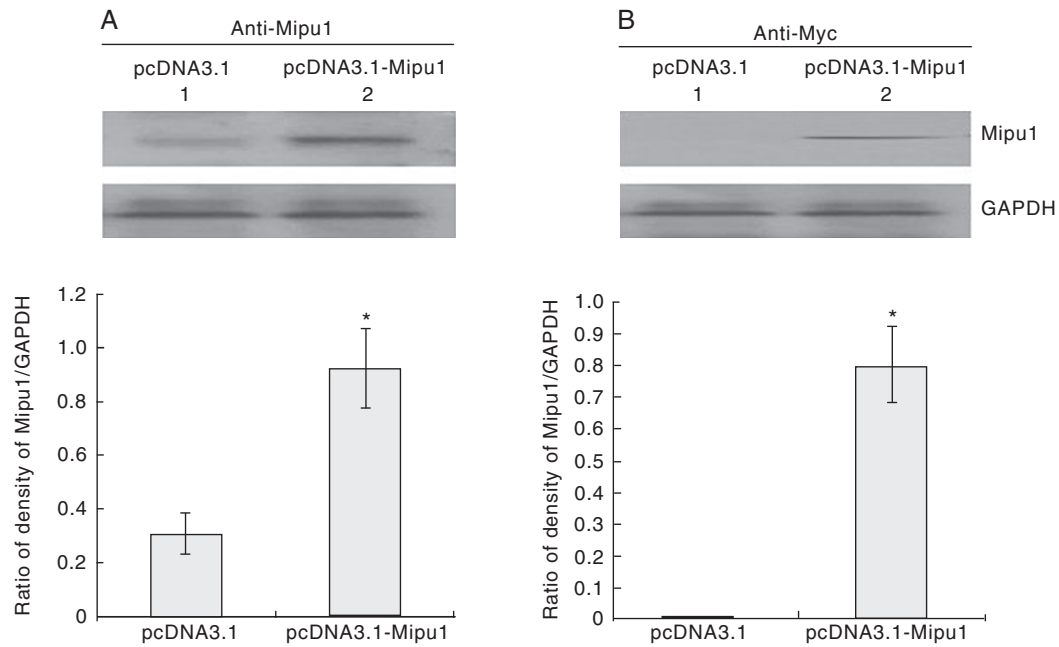


Figure 4. Expression of Mipu1 protein in H9c2 myogenic cells. *A*, Expression of exogenous Mipu1 protein from the pcDNA3.1-Mipu1 plasmid in H9c2 myogenic cells identified by the anti-Mipu1 polyclonal antibody. *Lane 1*, H9c2 myogenic cells transfected with pcDNA3.1 plasmid; *lane 2*, H9c2 myogenic cells transfected with pcDNA3.1-Mipu1 plasmid. *B*, Expression of the exogenous Mipu1 protein from the pcDNA3.1-Mipu1 plasmid in H9c2 myogenic cells identified by the anti-Myc monoclonal antibody. *Lane 1*, H9c2 myogenic cells transfected with pcDNA3.1 plasmid; *lane 2*, H9c2 myogenic cells transfected with pcDNA3.1-Mipu1 plasmid. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. * $P < 0.05$ vs pcDNA3.1, $N = 5$ (two-tailed Student *t*-test).

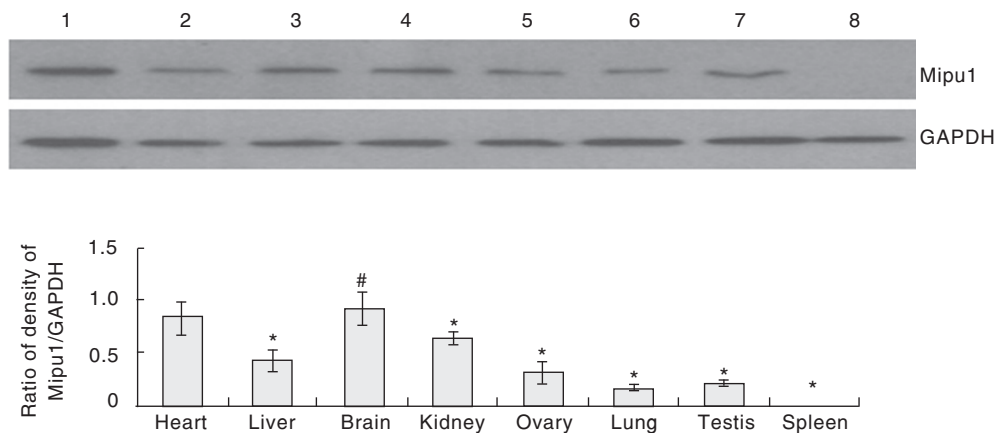


Figure 5. Expression of Mipu1 protein in rat tissues. The highest levels of Mipu1 protein were detected in the brain and heart, moderate levels were detected in liver, kidney, lung, ovary, and testis and low levels in the spleen. *Lane 1*, heart; *lane 2*, liver; *lane 3*, brain; *lane 4*, kidney; *lane 5*, ovary; *lane 6*, lung; *lane 7*, testis; *lane 8*, spleen. The relative values of all results were determined and are reported as mean \pm SEM of three experiments in duplicate. GAPDH = glyceraldehyde-3-phosphate dehydrogenase. * $P < 0.05$ vs heart; # $P > 0.05$ vs heart ($N = 5$; two-tailed Student *t*-tests).

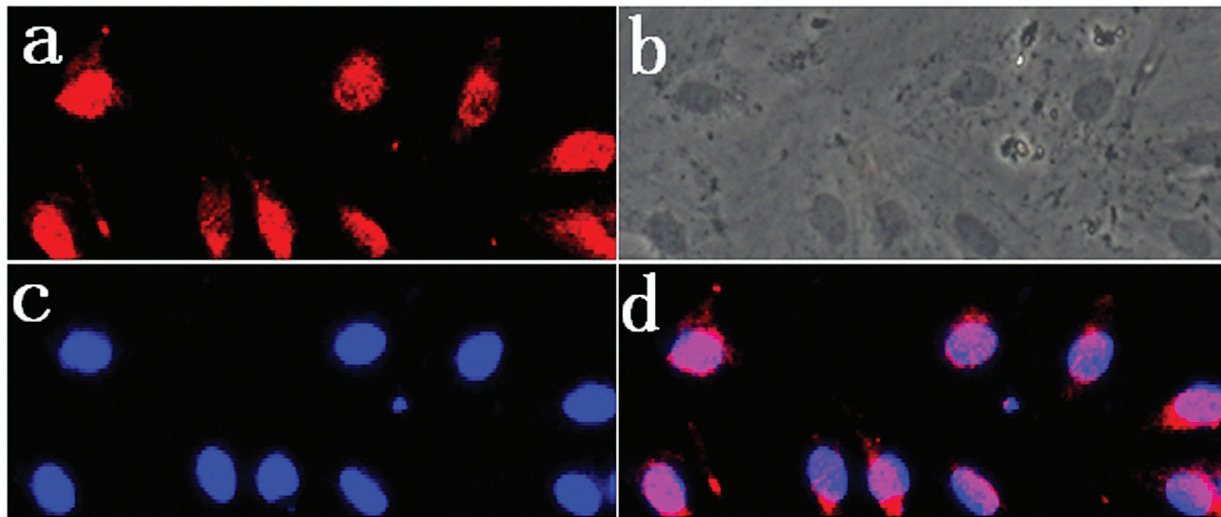


Figure 6. Sub-cellular localization of Mipu1 in H9c2 myogenic cells shown by indirect fluorescent immunocytochemistry. *a*, Endogenous Mipu1 protein in H9c2 myogenic cells stained first with the anti-Mipu1 polyclonal antibody and then with CY3-conjugated goat anti-rabbit IgG (red). *b*, Micromorphous H9c2 myogenic cells. *c*, Nuclei labeled with Hoechst 33258 (blue). *d*, Merged image of A and C (pink).

Sub-cellular localization of Mipu1

Fluorescent immunocytochemistry showed that Mipu1 protein was localized in the nucleus of H9c2 myogenic cells (Figure 6).

Discussion

The ability to achieve site-specific correction or modification of the genome has widespread implications for basic and applied research. Dion et al. (6) confirmed that the individual zinc finger domain recognizes DNA triplets with high specificity and affinity. By fusing zinc fingers to repression or activation domains, genes can be selectively targeted and switched off and on. The family of Kruppel-like proteins is one of the largest families of zinc finger proteins. These proteins contain two or more C_2H_2 -type zinc fingers that are separated by a conserved consensus sequence, T/SGEKPY/FX. The C_2H_2 type zinc finger motif was initially found in TFIII, a transcription factor of *Xenopus*, and subsequently in the Kruppel of *Drosophila* (7). Members of the Kruppel-like zinc finger family can function as activators or repressors of gene transcription and regulate embryonic development as well as a variety of physiological processes (8,9). Recently, studies focusing on C_2H_2 type zinc finger genes have suggested their unique involvement in the regulation of embryogenesis and in a variety of diseases (10-13).

Mipu1 is a novel gene that was found to be up-regu-

lated in rat heart after a transient myocardial ischemia-reperfusion procedure. The Mipu1 gene was composed of five exons and four introns with an open reading frame of 1827 bp, and mapped to chromosome 1q12.1, encoding a 608 amino acid polypeptide with an N-terminal KRAB domain and 14 C-terminal C_2H_2 zinc fingers. Jiang et al. (5) used a Mipu1 fusion protein, GST-Mipu1, immobilized on glutathione-bound Sepharose beads to capture specific oligonucleotide sequences from a random oligonucleotide library. These studies confirmed that Mipu1 could bind specifically to the consensus sequence 5'-TGTCTTATCG AA-3', with TCTTA as the core sequence, which suggested that Mipu1 might act as a transcriptional repressor.

In order to prepare the anti-Mipu1 antibody for the future study of the function of Mipu1, the recombinant prokaryotic expression vector pQE31-Mipu1 was constructed and transfected into *E. coli* M15(pREP4). The Mipu1-6His fusion protein was induced to express in *E. coli* M15(pREP4) and optimized to maximize yield by adding 1 mM IPTG at A600 of 0.6 after a series of trials of different concentrations of IPTG and different induction times. Next, the bacterial cell pellet was purified with a His-bind resin after being schizolyzed with lysozyme. The Mipu1-6His fusion protein was at least 85% purity after being purified by the His-bind resin and attained at least 98% purity after being further purified with ion-exchange resin. Analysis of the purified Mipu1-6His fusion protein using SDS-PAGE and Western blotting indicated that the purified protein migrated with a

mobility of 70 kDa, which corresponded to the molecular weight of Mipu1 protein. The identity of the purified Mipu1-6His fusion protein was further confirmed by MS/MS. We then used the purified Mipu1-6His fusion protein to immunize New Zealand rabbits and produced anti-Mipu1 antiserum. After the titer of the anti-Mipu1 antiserum was found to be satisfactory by ELISA, the rabbits were bled and the serum collected and purified using an Ab buffer kit. The polyclonal anti-Mipu1 polyclonal antibody was able to bind specifically to Mipu1 by Western blotting, which indicated that the antibody was specific and sensitive as a tool for further study of Mipu1.

Accordingly, using the anti-Mipu1 polyclonal antibody we found that H9c2 myogenic cells could express endogenous Mipu1 protein and exogenous Mipu1 protein from the pcDNA3.1-Mipu1 plasmid. We further observed the expression of Mipu1 in various rat tissues and found that Mipu1 was highly expressed in brain and heart, which suggested that Mipu1 might exert special functions in the brain and heart. Using fluorescent immunocytochemistry, we further found that the Mipu1 protein was localized to the nucleus of H9c2

myogenic cells, in agreement with the report of Jiang et al. (5) using a GFP tag. This study was coincident with the finding that Mipu1 is a kind of intranuclear protein and probably acts as a transcription factor. According to our previous study, which showed that C₂C₁₂ myogenic cells transfected with pcDNA3.1-Mipu1 were more resistant to oxidative injury compared to control, we can infer that Mipu1 is probably involved in endogenous cardioprotection against myocardial ischemia-reperfusion and oxidative injury by regulating the expression of some target genes. More studies are needed to clarify the functions of Mipu1 under physiological and pathological conditions.

In the present study, Mipu1 was highly expressed and purified, and a highly specific and sensitive polyclonal antibody against Mipu1 was produced. The antibody could be used as a tool to explore the function of Mipu1. Localized in the nucleus, Mipu1 was expressed in H9c2 myogenic cells and in most rat tissues, with an especially high level in the brain and heart. The present study provides the foundation for further studies on the biological functions of Mipu1.

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