Identification, Characterization, and Functional Analysis of Heart-specific Myosin Light Chain Phosphatase Small Subunit*

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Myosin light chain phosphatase consists of three subunits, a 38-kDa catalytic subunit, a large 110–130-kDa myosin binding subunit, and a small subunit of 20–21 kDa. The catalytic subunit and the large subunit have been well characterized. The small subunit has been cloned and studied from smooth muscle, but little is known about its function and specificity in the other muscles such as cardiac muscle. In this study, cDNAs for heart-specific small subunit isoforms, hHS-M21, were isolated and characterized. Evidence was obtained from an analysis of genome to suggest that the small subunit was the product of the same gene as the large subunit. Using permeabilized renal artery preparation and permeabilized cardiac myocytes, it was shown that the small subunit increased sensitivity to Ca2+ in muscle contraction. It was also shown using an overlay assay that hHS-M21 bound the large subunit. Mapping experiments demonstrated that the binding domain and the domain involved in the increasing Ca2+ sensitivity mapped to the same N-terminal region of hHS-M21. These observations suggest that the heart-specific small subunit hHS-M21 plays a regulatory role in cardiac muscle contraction by its binding to the large subunit.

Ca2+ plays a central role in the regulation of muscle contractile process mediated by interaction of myosin with actin. It is well known that phosphorylation status of myosin light chain (MLC) is correlated with the Ca2+ sensitivity of muscle contraction (1, 2). Two key enzymes regulate the phosphorylation status of MLC; myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). The functional role of MLCK has been well investigated (3, 4), whereas that of MLCP and related topics has only recently been expanding (5, 6).

A frequently used classification of protein phosphatases identifies two classes, type 1 (PP1) and type 2 (PP2) (7). In general, PP1 holoenzymes consist of a catalytic subunit (PP1c) and different regulatory subunits that may target the phosphatase to particular substrates (8, 9). MLCP is classified into PP1 (10) and composed of approximately 38-kDa PP1c and regulatory subunits in the smooth muscle (11–13). There are two different regulatory subunits, large and small, of about 110–130 kDa (myosin binding subunit, MBS) and 20 kDa (sm-M20), respectively (5, 6).

MBS cDNAs have been cloned from various tissues of chicken, rat, and human (5, 6), while cDNA for small subunit (sm-M20) has so far been isolated only from chicken gizzard (14). Because MBS has a regulatory role or a targeting function (5, 6), the gene for MBS was designated as the myosin phosphatase targeting subunit gene, MYPT1 (15). MBSs encoded by MYPT1 from various species have a common structural feature such as ankyrin repeats in the N-terminal half of the molecule, and those from human and rat have leucine zipper motifs in the C-terminal end. It has been reported that MBS binds to PP1c, myosin heavy chain (MHC), and MLC to form an MLCP complex (12, 16–20) and that both MHC and MLC are dephosphorylated in this MLCP complex (16–18). The dephosphorylation of myosins by PP1c is enhanced by the presence of MBS, and thereby, the function of MBS is to up-regulate the activity of PP1c toward the myosins (16–18).

In contrast, only a limited amount of information is available for sm-M20. It also contains C-terminal leucine zipper motifs and is homologous to the C-terminal one-third of MBS (14). It has been reported that sm-M20 interacts with myosin and the C-terminal end of MBS (18, 19). In addition, we have recently demonstrated that sm-M20 increases the Ca2+ sensitivity of the contractile apparatus in vascular smooth muscle and that this effect was conferred by the N-terminal half of sm-M20 (21). However, the existence and function, if any, of M20 subunit in other muscles than the smooth muscle remain unknown.

Several protein phosphatases in the cardiac muscle have...
been characterized (14, 22, 23). There are, however, only a few reports about the MLCP in the cardiac muscle (24, 25). In addition, it has been considered that the Ca$^{2+}$ sensitivity of contraction in the striated muscle is mainly regulated by troponins (26), and the significance of MLC phosphorylation in the Ca$^{2+}$ sensitization of the striated muscle remains to be elucidated. However, a recent report has demonstrated a possible involvement of MLC phosphorylation in the development of cardiac hypertrophy (27).

Recently, another gene for MBS, MYPT2, was isolated by Fujioka et al. (25). Toward the N terminus and C terminus, MBS encoded by MYPT2 has seven ankyrin repeats and three leucine zipper motifs, respectively, which are highly homologous to the relevant sequences of MBS encoded by MYPT1. Because MYPT2 is expressed preferentially in the striated muscles, especially in the cardiac muscle, it was suggested that the function of MYPT2 might be related with the regulation of MLC phosphorylation in the heart (25). The functions of MLC and MLCP in the cardiac muscle, then, should be unraveled for better understanding of the regulation of muscle contractility in the heart.

We report here the isolation of cDNAs for heart-specific isoform of M20 subunit (hHS-M21), which were obtained in the MLCP phosphorylation in the heart (25). The functions of MLC muscles, especially in the cardiac muscle, it was suggested that the regulation of MLC phosphorylation in the cardiac muscle. From an analogy of function for sm-M20 contraction was investigated, along with its interaction with MYPT2.

We, then, determined genomic structure of the gene for hHS-M21 and analyzed the expression profiles of hHS-M21. In addition, the remaining regions, a long amplification PCR (LA-PCR) method was used to determine the sequences of each exon and adjacent introns.

**RT-PCR Analyses of MYPT2 and MYPT1—**Tissue-specific expression patterns of hHS-M21 gene (corresponding to HS602 cDNAs) and that of MBS gene (MYPT2) were investigated by RT-PCR analyses using mRNAs from human tissues. The primers used for the RT-PCR analyses had the following sequences: 602F (5'-TATGCAAAGGACATGCAAGG-3'), 602-EX6R (5'-GCTTTCAAGGAGTCTGTAAC-3'), 602-EX7R (5'-GGACATGGTGAGTTTTCTGGTGGA-3') and 602-EX11R (5'-AGAGATGAAAGGGCTGGTGGA-3'). The LA-PCR products were electrophoresed in 0.6–0.8% agarose gels and extracted from the gels using a gel Band Purification Kit (Amersham Pharmacia Biotech). The purified DNA fragments were sequenced from multiple scanning primers designed in exons of MYPT2 to determine the sequences of each exon and adjacent introns.

**Isolation of a Heart-specific cDNA—**To obtain information on genes preferentially expressed in the cardiac muscle, a normalized subtraction PCR-cDNA library between mRNAs of cardiac and skeletal muscles was constructed using a PCR-Select cDNA subtraction kit (CLONTECH). Randomly selected clones from this library were determined for their nucleotide sequences in the GenBank database. Several cDNA fragments that were found more than two times in the sequenced cDNA fragments and had no identity in the data base except for human expressed sequence tags were investigated for tissue specificity of gene expression using RT-PCR analysis. The RT-PCR analysis, mRNA from various human tissues including fetal heart, adult heart, skeletal muscle, brain, kidney, liver, uterus, lung, spleen, thymus, small intestine, and colon (CLONTECH) were examined. A cDNA fragment (HS602) that was expressed only in the heart was investigated further. To isolate full-length cDNA clones encoding the HS602 gene, a human heart cDNA library in agt11 (CLONTECH) was screened using HS602 fragment as a probe according to the standard methods (28). The cDNA inserts from positive recombinant phages were subcloned into plBlueScript KS (−) (Invitrogen) for sequence determination.

**Determination of Genomic Organization of MYPT2—**Several clones containing a part of MYPT2 were isolated from a human genomic DNA library in EMBL3 (CLONTECH) using the longest cDNA clone for the HS602 gene, 602-7, as a probe. Isolated genomic DNA clones were subcloned into plBlueScript KS (−) and the subclones hybridized to 602-7 were sequenced. These subclones were revealed to contain exons 14–18, 20, 21, and 23–25 of MYPT2. To obtain sequence information for the remaining regions, a long amplification PCR (LA-PCR) method was performed using an LA-PCR kit (Takara) according to the manufacturer’s instructions. The primer pairs used in the LA-PCR experiments were as follows: MYPT2LA1F (5'-GGAACATGGCAGAGAGATGAATT-3') and MYPT2LA1AR (5'-CCTTCTGCCTGCTGCTTCCATT-5'); MYPT2LA1AFP (5'-GGACATGGCAGAGAGATGAATT-3') and MYPT2LA1AR (5'-CCTTCTGCCTGCTGCTTCCATT-5'); MYPT2LA2F (5'-GGACATGGCAGAGAGATGAATT-3') and MYPT2LA2AR (5'-CCTTCTGCCTGCTGCTTCCATT-5'); MYPT2LA2F (5'-GGACATGGCAGAGAGATGAATT-3') and MYPT2LA2AR (5'-CCTTCTGCCTGCTGCTTCCATT-5'); MYPT1LA4F (5'-CTTCATGGCGGCGGTCCTGACA-3') and MYPT1LA4R (5'-CTTCATGGCGGCGGTCCTGACA-3'), MYPT2LA4F (5'-CTACTCCTGCTGCCTCATT-3') and HSpromR5 (5'-CTGCGCATTCTTGGCTGTC-3'), 602-EX6f (5'-GGCTTCAAGGGATGTGGTGAC-3') and 602-EX7R (5'-CGAAGATTCAGAGTGGG-3'), MYPT2LA3F (5'-CTCCTGCGGCTGAGTGGG-3') and MYPT2LA3R (5'-CTCCTGCGGCTGAGTGGG-3') and 602-EX11R (5'-AGAGATGAAAGGGCTGGTGGA-3'). The LA-PCR products were electrophoresed in 0.6–0.8% agarose gels and extracted from the gels using a gel Band Purification Kit (Amersham Pharmacia Biotech). The purified DNA fragments were sequenced from multiple scanning primers designed in exons of MYPT2 to determine the sequences of each exon and adjacent introns. The RT-PCR products were electrophoresed in 1.4% agarose gel. For comparison of the mRNA expression levels of MYPT1 and MYPT2, RT-PCR analyses were performed for mRNAs from six human tissues, heart, skeletal muscle, brain, uterus, lung, and small intestine. The sequences of primers used were as follows; MYPT1-1-40F (5'-AAGGAGC-3'), MYPT1-1-40R (5'-CTCCTGCGGCTGAGTGGG-3'), 602-EX6f (5'-GGCTTCAAGGGATGTGGTGAC-3') and 602-EX7R (5'-CGAAGATTCAGAGTGGG-3'). The LA-PCR products were electrophoresed in 1.4% agarose gel. Expression and Purification of Recombinant Proteins—Various fragments of the hHS-M21 cDNA were expressed as fusion proteins in a prokaryote expression system (Qiagen) and long amplification PCR (LA-PCR) method. The insert was excised by digestion with EcoRI and BamHI and cloned into the EcoRI BamHI-cleaved pQE30 vector in which the hemagglutinin tag was replaced by a flag tag. The pQE30 flag vector was constructed as follows. At first, PCR amplification with primers, 5'-CTCTTGGCTTCTCACCAGGAG-3' (sense) and 5'-GATGTCGGC-3', and MYPT2-600F (5'-GAGCTTCCGGGCTGGTACG-3'), MYPT2-600R (5'-GGACATGGTGGTTCGTCACG-3'). The PCR products were then cloned into pCR2.1 (Invitrogen) for sequence confirmation and the insert cDNAs were excised by digestion with EcoRI and BamHI and cloned into the EcoRI BamHI-cleaved pQE30 to obtain pQ30 flag vector.

The recombinant hHS-M21 proteins generated were hHS-M21-A and hHS-M21-B (two constructs for major isoforms; corresponding to exons 16–25 or exons 16–24 of MYPT2, respectively), hHS-M21-t (deletion of N-terminal 56 residues and leucine zipper motifs; corresponding to exons 16–20), hHS-M21-t-2 (N-terminal half of hHS-M21; exons 16–20), hHS-M21-t-3 (deletion of N-terminal 56 residues and leucine zipper motifs; exons 19–23), t-4 (C-terminal half of hHS-M21-A; exons 21–23), t-5 (C-terminal half of hHS-M21-B; exons 21–24), hHS-M21-o-1 (N-terminal 56 residues of hHS-M21; exons 16–18), and hHS-M21-o-2 (deletion of N-terminal 56 residues and C-terminal half of hHS-M21, exons 19–24). On the other hand, MYPT2-o-1 and MYPT2-o-2 were expressed as divided into three parts. The recombinant MBS protein encoded by MYPT2 were obtained as flag-tagged fusion proteins using a pQE30-flag vector in which the hemagglutinin tag was replaced by a flag tag. The pQE30 flag vector was constructed as follows. At first, PCR amplification with primers, 5'-CTCTTGGCTTCTCACCAGGAG-3' (sense) and 5'-GATGTCGGC-3', and MYPT2-600F (5'-GAGCTTCCGGGCTGGTACG-3'), MYPT2-600R (5'-GGACATGGTGGTTCGTCACG-3'). The PCR products were then cloned into pCR2.1 (Invitrogen) for sequence confirmation and the insert cDNAs were excised by digestion with EcoRI and BamHI and cloned into the EcoRI BamHI-cleaved pQE30 to obtain pQ30 flag vector.
K$_2$HPO$_4$ (pH 7.4)) and stored at -80°C until preparation of recombinant proteins.

The preparation of recombinant proteins from the cell pellets was done as follows. After thawing at 4°C, cells were sonicated in 6 mM urea, 5 mM imidazole, 500 mM NaCl, 5 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 20 mM Tris-HCl (pH 7.5) (buffer A) and centrifuged at 10,000 x g for 15 min at 4°C. Supernatants containing recombinant MBS proteins were stored at -80°C until use in binding assay. On the other hand, the supernatants containing recombinant hHS-M21 proteins were loaded into columns in which the resin (Nova-Gen) charged with Ni$^{2+}$ and equilibrated with buffer A was bedded. After the columns were washed extensively with 6 mM urea, 60 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl (pH 7.5), the eluates were dialyzed against 0.1% acetic acid (pH 4.0), and the dialysates were clarified by centrifugation at 10,000 x g for 15 min at 4°C. The supernatants were freeze-dried and dissolved in 0.1% acetic acid (pH 4.0) for concentration. The recombinant hHS-M21 proteins were further purified by using an HPLC system consisting of a pump (LC-10AD; Shimazu), a column (TSKgel G2000SWXL; Tosasa), and an UV detector (SPD-10AVP; Shimazu).

For Western Analysis—Equal amounts of recombinant MBS proteins were applied to SDS-PAGE in 12% acrylamide gels, and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% nonfat dry milk, incubated for 4 h at room temperature with hexahisidine-tagged hHS-M21 recombinant proteins (A, B, or t-1) solubilized in 20 mM PIPES-KOH (pH 7.1). After washing twice with PBS and twice with TBS, the membranes were incubated with horseradish peroxidase-conjugated rabbit anti-His6 tag polyclonal antibody (Santa Cruz) for 1 h. The immunocomplex was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) and AB1373A automated DNA sequencer. SDS-PAGE and the immunoblotting technique were carried out according to the standard procedures (36, 37). Concentration of protein was measured by the Bradford method (38) with bovine serum albumin (Pierce) as standard.

Data Analysis—The extent of force development was expressed as a percentage, assigning values in Ca$^{2+}$-free buffer (resting state) and in 10 μM Ca$^{2+}$ buffer (maximum contraction) to be 0% and 100%, respectively. The EC$_{50}$ value, a concentration of Ca$^{2+}$ required to induce the 50% force of the maximum response, was determined by fitting the Ca$^{2+}$ concentration-tension response curves to a four-parameter logistic model (39). The measured values were expressed as means ± S.E. Effects of recombinant hHS-M21 proteins on Ca$^{2+}$-induced force were statistically analyzed by analysis of variance. The obtained EC$_{50}$ values from pCa-tension relations were compared in the absence or presence of the hHS-M21 fragments using Student's t test for paired values, and the P values of less than 0.05 were considered to be statistically significant.

RESULTS

Isolation of hHS-M21 cDNAs—We constructed a normalized subtraction PCR-cDNA library between the mRNAs from cardiac and skeletal muscles and the heart-specific cDNA fragments. Randomly selected 1,021 clones were sequenced, and the sequence data were compared with the GenBank data base. It was revealed that 243 (23.8%) clones were not matched to the known human genes in the data base except for expressed sequence tags. Forty-seven cDNA fragments from unmatched genes were tested for their expression in various human tissues by the RT-PCR analysis. Among them, three cDNA fragments were expressed only in the heart (data not shown),
and one of these heart-specific clones, which were 209 bp of length, was designated as HS602.

The HS602 fragment was used as a probe to isolate cDNA clones from a human heart cDNA library. Restriction mapping and end sequencing of the isolated cDNAs showed that there were at least three types of cDNAs different by a small deletion or insertion. Representative clones of each type, 602-4, 602-6, and 602-7, were completely determined for their sequences, and it was revealed that 602-6 and 602-7 had an insertion of 181 bp and a deletion of 3 bp, respectively, as compared with 602-4 (Fig. 1). From an estimation by the numbers of isolated cDNA clones corresponding to these three types, it was suggested that two of them, 602-4 type and 602-6 type, were major isoforms and another type, 602-7 type, was a minor isoform.

Full sequences of HS602 cDNAs for two major isoforms were determined from the isolated overlapping clones. These two isoforms span a total of 2,046 and 2,227 bp with the open reading frame predicting to encode for 208 and 224 amino acid residues, respectively (Fig. 2). The full sequence included 827 bp in the 5'-untranslated region and 595 or 731 bp in the 3'-untranslated region for two different termination codons in the HS602 cDNAs.

Characteristic feature of the predicted proteins was the presence of leucine zipper motifs at the C-terminal end. Although the long isoform, hHS-M21 B, had different C-terminal structures by the 181-bp insertion from the short isoform, hHS-M21 A, leucine zipper motifs were found in both isoforms (Fig. 2). A database search revealed that the amino acid sequence of hHS-M21 A was 76.8% identical to the 20-kDa smooth muscle small subunit (sm-M20) of chicken myosin phosphatase (14) and 50.9% identical to C-terminal part of MBS (MYPT1) of human myosin phosphatase (15). Of particular interest was that the C-terminal half sequence of hHS-M21 A was 92.4% and 69.6% identical to the relevant sequence of sm-M20 and MBS (MYPT1), respectively. It was, then, suggested that the HS602 gene encoded for a heart-specific 21-kDa small subunit (hHS-M21) of human myosin phosphatase, and that the hHS-M21 had two major isoforms; the short type (hHS-M21 A) and the long type (hHS-M21 B). To our surprise, hHS-M21 A cDNA sequence was identical to 3'-terminal one-third sequence of MYPT2 cDNA for another MBS (25), except that 5'-sided 355-bp sequence of hHS-M21 cDNA was lacked from the MYPT2 cDNA. In addition, the initiation codon of hHS-M21 was corresponding to codon 775 of MYPT2, i.e. aa 1–208 of hHS-M21 A was exactly identical to aa 775–982 of MBS encoded by MYPT2. These observations strongly suggested that the MYPT2 gene was a multi-functional gene encoding for both the striated muscle type MBS and heart-specific M21. To obtain a structural evidence of this multicoding feature, we determined the genomic organization of the human MYPT2 gene.

**Genomic Organization of Human MYPT2 Gene**—To determine the genomic organization of the human MYPT2 gene, a human genomic DNA library was screened with 602-7 as a probe. Several different genomic clones were obtained and EcoRI or XhoI fragments hybridized to 602-7 were subcloned for the sequencing analyses from primers designed in the MYPT2 cDNA sequence. Representative genomic clones, 602G2, 602G4, 602G3, and 602G1, are shown in Fig. 3 along with their subclones, 602gs2, 602gs7, 602gs4, 602gs3, 602gs1, 602gs11, and 602gs10, containing exons 14–18, 20, 21, and 23–25. Because the screening of genomic DNA library has failed to isolate clones corresponding to the other exons, the remaining genomic organization was determined by sequencing of overlapping LA-PCR products (Fig. 3).
Sequencing analyses of genomic subclones and LA-PCR products have revealed that the MYPT2 gene consists of 25 exons as shown in Fig. 3 and Table I. The hHS-M21 B cDNA was the product of alternative splicing at exon 24, and the 3-bp deletion in CDNA clone 602-7 was suggested to be a differential splicing product to a minor acceptor site in exon 22 (Table I). It also was revealed that the ankyrin repeats in the N-terminal part of MBS (MYPT2) molecule were encoded by exons 1–7, and two mutually exclusive leucine zipper motifs at the C-terminal part of the hHS-M21 A and B cDNAs were encoded by exon 25 and 24, respectively (Fig. 3). Quite interestingly, the 5’ sequence (1–355 bp) of hHS-M21 cDNA (exon 1 of hHS-M21) was found in intron 13 (1 kbp upstream of the exon 14) of the MYPT2 gene and exons 14–25 were exactly matched to the hHS-M21 sequences (Fig. 3). In addition, several cis-elements that were observed in promoter regions of heart-specific genes, GATA binding site, MEF-2-like binding motif, E-box, AP2 and two mutually exclusive leucine zipper motifs at the C-terminal part of the hHS-M21 A and B cDNAs were encoded by exon 25 and 24, respectively (Fig. 3). Quite interestingly, the 5’ sequence (1–355 bp) of hHS-M21 cDNA (exon 1 of hHS-M21) was found in intron 13 (1 kbp upstream of the exon 14) of the MYPT2 gene and exons 14–25 were exactly matched to the hHS-M21 sequences (Fig. 3). In addition, several cis-elements that were observed in promoter regions of heart-specific genes, GATA binding site, MEF-2-like binding motif, E-box, AP2 and two mutually exclusive leucine zipper motifs at the C-terminal part of the hHS-M21 A and B cDNAs were encoded by exon 25 and 24, respectively (Fig. 3). Quite interestingly, the 5’ sequence (1–355 bp) of hHS-M21 cDNA (exon 1 of hHS-M21) was found in intron 13 (1 kbp upstream of the exon 14) of the MYPT2 gene and exons 14–25 were exactly matched to the hHS-M21 sequences (Fig. 3).

Expression of MYPT1, MYPT2, and hHS-M21 in Human Tissues—Expression of MYPT2 (this means MBS coding region of the MYPT2 gene here) and hHS-M21 in various human tissues was investigated using the RT-PCR analysis and compared with that of MYPT1 (MBS coding region of the MYPT1 gene). As shown in Fig. 4A, hHS-M21 mRNA was expressed only in the heart, while MYPT2 expression was detected in several tissues, preferentially in heart, skeletal muscle, and brain. It was confirmed by the RT-PCR analysis that exon 24 of the MYPT2 gene was alternatively spliced in encoding for the MBS molecule (Fig. 4A), as is the case for encoding for the hHS-M21 molecule (Fig. 1). Expression of mRNA skipped for exon 24 was relatively abundant as compared with mRNA utilizing exon 24 in most tissues, whereas similar quantities of both type mRNA were found in the skeletal muscle (Fig. 4A).

A competitive RT-PCR analysis showed the different expression level of MYPT1 and MYPT2 in human tissues (Fig. 4B). The expression of MYPT2 mRNA was higher than that of MYPT1 in the striated muscles, such as heart and skeletal muscle. In contrast, the amount of MYPT1 mRNA was more abundant than that of MYPT2 mRNA in brain and other tissues including lung, uterus, and small intestine. It should be noted here that the expression level of MYPT1 mRNA was relatively constant but somewhat different in tissues tested here (Fig. 4B).

Effect of hHS-M21 Fragments on the Ca2+ Sensitivity of Contraction in Permeabilized Porcine Renal Artery and Rat Cardiac Myocytes—To analyze the function of hHS-M21 in muscle contraction, various recombinant hHS-M21 proteins were prepared (Fig. 5A) and used in permeabilized cell assays. Fig. 5 (B and C) shows the effect of hHS-M21 proteins on the Ca2+-induced contraction in 1% Triton X-permeabilized porcine renal artery and 2.5% β-escin-permeabilized rat cardiac myocytes, respectively. In these protocols, contractions were monitored by stepwise increases in Ca2+ concentration in the presence of 3 μM recombinant hHS-M21 proteins in permeabilized porcine renal artery and 1 μM recombinant hHS-M21 proteins in permeabilized rat cardiac myocytes. In the Ca2+-free buffer, application of hHS-M21 even up to 10 μM, was not able to produce any significant tension development in both permeabilized porcine renal artery and rat cardiac myocytes (data not shown). Then, the observed contraction reflected the Ca2+ sensitization effects by the hHS-M21 proteins.

In permeabilized porcine renal artery, application of 3 μM recombinant proteins, hHS-M21 23 A, hHS-M21 23 B, and hHS-M21 t-1, caused augmentation of the Ca2+-induced contractions, and the [Ca2+] force relation curves were shifted to left as compared with that in the controls where no recombinant proteins were added (Fig. 5B). The EC50 value of Ca2+ in the control assay was 434.8 ± 17.4 nM (n = 3). In the presence of 3 μM recombinant proteins, hHS-M21 23 A, hHS-M21 23 B, and hHS-M21 t-1, the EC50 values were significantly lowered to 86.9 ±
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Table I

DNA sequences around each exon/intron boundary are shown. Position and length of each intron are indicated, along with the corresponding codons for exons. Two alternative splicing acceptor site sequences at the junction of intron 21 are underlined.

| Preceding intron | Exon (length of exon) | Following intron | Length of following intron | Corresponding codons for exon |
|------------------|----------------------|-----------------|---------------------------|-----------------------------|
| GAGGAT CC. Exon 1 (454 bp) ..CAC... | gtaactctc... | >6 kbp | 1-97 |
| GCCA TGT. Exon 2 (131 bp) ..CCA... | gtagtgagtt... | 6.0 kbp | 98-140 |
| GAT TAT. Exon 3 (119 bp) ..AAA... | gtaacctctat... | 3.5 kbp | 141-180 |
| GA TTT. Exon 4 (160 bp) ..CTC... | gtaattgcca... | 1.4 kbp | 181-233 |
| ACT TTA. Exon 5 (145 bp) ..AAA... | gtaattgatcc... | 1.4 kbp | 234-282 |
| GAG CAG. Exon 6 (75 bp) ..AAT... | gtagcttgttt... | 2.0 kbp | 283-337 |
| GAG CTA. Exon 7 (80 bp) ..AGG... | gtaagatttt... | 0.8 kbp | 308-337 |
| AAA GGA. Exon 8 (140 bp) ..GCA... | gtaatgcaaa... | 3.4 kbp | 334-380 |
| AT AAA. Exon 9 (113 bp) ..AGG... | gtagctacttt... | 3.0 kbp | 381-418 |
| TTC TCT. Exon 10 (204 bp) ..GAT... | gtagccagttt... | 3.3 kbp | 419-468 |
| GAG AGA. Exon 11 (83 bp) ..AAC... | gtaacctcaaa... | 1.8 kbp | 487-513 |
| A AA. Exon 12 (126 bp) ..AAA... | gtagaagcgc... | 3.1 kbp | 514-555 |
| ACT G. Exon 13 (183 bp) ..AGG... | gtagtccaa... | -20.0 kbp | 556-616 |
| G TCC. Exon 14 (91 bp) ..ACT... | gtagtggtgg... | 5.0 kbp | 617-647 |
| GGT GTC. Exon 15 (204 bp) ..GAA... | gtagctctat... | 1.9 kbp | 648-715 |
| CCT ACT. Exon 16 (190 bp) ..AAT... | gtagtgcaa... | 87 bp | 716-773 |
| AG AAT. Exon 17 (113 bp) ..AAA... | gtagagtggat... | 1.2 kbp | 779-816 |
| GAG GAT. Exon 18 (42 bp) ..AGC... | gtagatgca... | >40 kbp | 817-830 |
| CAT GAA. Exon 19 (117 bp) ..TCT... | gtaagaacctt... | 3.8 kbp | 831-835 |
| G TTG. Exon 20 (145 bp) ..AAA... | gtaggtctttt... | 1.7 kbp | 836-884 |
| CCT TAT. Exon 21 (105 bp) ..GCC... | gtagacgcg... | 3.5 kbp | 885-919 |
| CAG AAA. Exon 22 (59 bp) ..AAA... | gtagctgcaag... | 1.5 kbp | 920-937 |
| GAG AGG. Exon 23 (51 bp) ..ATG... | gtagaagag... | 6.1 kbp | 938-954 |
| CTC TAT. Exon 24 (181 bp) ..TAG... | gtagtttctcag... | 6.0 kbp | 955-998 |
| AAC CTC. Exon 25 (679 bp) ..TAG... | gtagctcag... | 955-982 |

2.0 nM, 94.0 ± 7.1 nM, and 118.4 ± 2.4 nM, respectively (for each case, n = 3, p < 0.001) (Table II).

In permeabilized rat cardiac myocytes, the [Ca2+]i, force relationships obtained in the presence of these recombinant proteins were shifted leftward (Fig. 5C), as observed in the permeabilized porcine renal artery. Although the EC50 value obtained in the control assay was 957.6 ± 169.3 nM (n = 5), it was significantly decreased to 318.9 ± 31.7 nM (n = 5, p < 0.01), 261.2 ± 31.6 nM (n = 5, p < 0.01), and 555.7 ± 90.1 nM (n = 5, p < 0.05) in the presence of 1 μM recombinant protein, hHS-M21 A, hHS-M21 B, and hHS-M21 t-1, respectively.

These results indicated that the exogenously added hHS-M21 proteins increase the Ca2+ sensitivity of the contractile apparatus in the permeabilized smooth muscle (porcine renal artery) and cardiac muscle (rat cardiac myocytes). The effect of hHS-M21 was prominent in the smooth muscle, but it was significantly observed also in the cardiac muscle. Because the presence or absence of C-terminal leucine zipper motifs showed a little but with no statistically significant difference in the effect of muscle contraction, it was suggested that the main functional domain of hHS-M21 was not located in the C-terminal leucine zipper motifs.

Interaction of hHS-M21 with MYPT1 and MYPT2—As demonstrated in the previous section, hHS-M21 showed a prominent effect in the smooth muscle and, to less extent, in the cardiac muscle. This phenomenon might be curious, because the hHS-M21 gene is expressed only in the cardiac muscle and not in the smooth muscle. However, it can be explained if hHS-M21 exhibits its function mainly through interaction with MBS encoded by MYPT1, and not with MBS encoded by MYPT2. To investigate the interaction of hHS-M21 with MBSs encoded by MYPT1 and MYPT2, an overlay assay was used to evaluate the binding affinity between them. Both isoforms of full-length hHS-M21 (A and B) showed a binding ability to C-terminal one-third of MYPT1-MBS (MYPT1 P), while its binding to the corresponding part of MYPT2-MBS (MYPT2 P) was extremely low (Fig. 6A). In addition, the hHS-M21 proteins did not bind to N-terminal two-thirds of MBS encoded by either MYPT1 or MYPT2 (Fig. 6A).

To further map the binding domain of hHS-M21 to the C-terminal one-third of MYPT1-MBS or MYPT2-MBS, various hHS-M21 fragments were tested in the overlay assay. The fragments containing the N-terminal 56 residues of hHS-M21 (t-1, t-2, and o-1) bound to the MYPT1-MBS with high affinity and to the MYPT2-MBS with extremely low affinity, as the full-length hHS-M21 proteins did. In contrast, the C-terminal hHS-M21 fragments (t-3, t-4, and t-5) showed only a scarce binding to MBSs (Fig. 6B) and no binding was observed with the middle part of hHS-M21 (o-2). These results also demonstrated that the C-terminal leucine zipper motifs of the hHS-M21 proteins were dispensable for the sufficient interaction with MBS encoded by MYPT1.

Effect of N- or C-terminal Fragments of hHS-M21 on Ca2+ Sensitivity—Because the hHS-M21 protein showed a high binding affinity to MYPT1-MBS via its N-terminal 56 residues and because the exogenously added full-length hHS-M21 proteins enhanced the Ca2+-induced muscle contraction, the effects of hHS-M21 fragments were investigated to map the main functional enhancing domain of hHS-M21. To determine the enhancing domain, we used the permeabilized porcine renal artery assay, because the effect of hHS-M21 was prominent in this assay as compared with that in the rat cardiomyocytes.

The [Ca2+]i force relationship obtained in the presence of 3 μM hHS-M21 recombinants, t-2 and o-1, was shifted leftward (data not shown). The EC50 values obtained in the presence of hHS-M21 t-2 and o-1 were significantly small as compared with that in the control (n = 3, p < 0.001 and p < 0.01, respectively), although the EC50 value for hHS-M21 t-2 was smaller than that for hHS-M21 o-1 (Table II). On the other hand, the [Ca2+]i force relationship in the presence of 3 μM hHS-M21 recombinants, t-3, t-4, and t-5, overlapped with that in the control (data not shown) and the EC50 values were not significantly different from the control value (Table II). Therefore, the main active domain of hHS-M21 was mapped in the similar region as the
binding domain, although the enhancing activity of o-1 (resi-
dues 1–56) was less than that of o-2 (residues 1–110).

**DISCUSSION**

In the present study, we isolated and characterized cDNA
clones for the human myosin light chain phosphatase (MLCP)
subunit (hHS-M21) that was expressed only in the heart. Genomic organization of the human MYPT2 gene was
determined, and it was revealed that one of the large subunits
(MYPT2-MBS) and the small subunit of MLCP in the heart are
the products of the same gene. In addition, we investigated the
function of hHS-M21 as measured by the Ca^{2+} sensitivity of
contraction in the permeabilized porcine renal artery and rat
cardiac myocytes. Moreover, the interaction of the hHS-M21
with MBSs encoded by MYPT1 and MYPT2 was investigated by
the overlay assay using recombinant proteins. We found that:
1) hHS-M21 induced an additional contraction at a con-
stant Ca^{2+} concentration and shifted the [Ca^{2+}]_i relationship
-towards the left; 2) the fragments containing the N-terminal half
of the hHS-M21, while the deletion of the N-terminal 56 residues completely abolished the action; 3) hHS-M21 bound to the C-terminal one-third of MBS encoded by
MYPT1 with high affinity, and only to a little extent, bound to
the same region of MBS encoded by MYPT2; and 4) the binding
domain of hHS-M21 to MYPT1-MBS was mapped to the same
region as the main effective domain for the Ca^{2+} sensitivity on
the Ca^{2+}-induced contraction in permeabilized porcine renal
artery.

The MLCP holoenzyme is composed of three subunits; cata-
lytic subunit and small and large regulatory subunits (5, 6).
The MYPT2 gene locates on chromosome 1q32 and encodes for
MBS of an approximately 125-kDa protein in the cardiac mus-
cle, of which sequence is 61% identical to that of the human
MYPT1 gene expressed in the smooth muscle (25). Unexpected-
edly, the C-terminal region (residues 775–982) of the MYPT2-
MBS was 100% identical to the full-length amino acid sequence
of the hHS-M21 A, one of the two major isoforms of human
hHS-M21, and moreover, residues 775–954 of the MYPT2-MBS
were 100% identical to residues 1–180 of the hHS-M21 B, an-
other isoform of human hHS-M21. These observations led us to
speculate that the hHS-M21 isoforms may be products of the
MYPT2 gene. To confirm this speculation, we carried out deter-
mination of genomic organization of the MYPT2 gene and
investigated their expressions by the RT-PCR analyses. It was
revealed that sequence of 5’-untranslated region of the hHS-
M21 cDNA was present in intron 13 of MYPT2, and that the two
major isoforms of human hHS-M21 were derived from the al-
ternative splicing of exon 24 of MYPT2 (Figs. 3 and 4). These
results have demonstrated that the C-terminal 208 (or 224)
residues of MYPT2-MBS are expressed in the heart as a sepa-
rate protein, termed as hHS-M21, of which mRNAs are tran-
scribed from a heart-specific promoter located within an intron
of the MYPT2 gene. We also have confirmed that the hHS-M21
protein is expressed in the human heart by a Western blot
analysis using rabbit antisera raised against the recombinant
hHS-M21 A protein (data not shown).

It has recently been suggested that the sm-M20 subunit from
chicken gizzard may be produced from an avian orthologue of
MYPT2 (40), while the sm-M20 subunit has not been isolated
yet from human tissue. However, the C-terminal 120 residues
of chicken sm-M20 (residue 67–186) showed 91% identity to the
C-terminal 120 residues of hHS-M21, whereas the homology in
N-terminal residues between chicken sm-M20 and hHS-M21 were only about 50%. It is of interest that intron 18 of the
human MYPT2 gene is long (more than 40 kbp) as is the case
with intron 13 (about 20 kbp) where hHS-M21 exon 1 exists and
that the high sequence similarity of hHS-M21 with chicken
sm-M20 was found after the sequence of exon 19. If mRNA of
human sm-M20 subunit were transcribed from intron 18 of the
MYPT2 gene, it would be a very unique gene that encodes for
different MLCP regulatory subunits: MBS, hHS-M21, and
sm-M20.

It might be unusual for one gene to produce two or more
proteins with different function in the same tissue by utilizing
different promoters, but MLCK gene is known to generate two
different proteins in the smooth muscle. The C-terminal 154
residues of the smooth muscle MLCK is expressed as an inde-
pendent protein, telokin (41, 42), and the telokin cDNA is tran-
scribed from a promoter within an intron of the MLCK
gene (42, 43). Telokin binds specifically to dephosphorylated
MLC of smooth muscle and inhibits MLC phosphorylation by
MLCK (44, 45). In addition, telokin induces Ca^{2+} desensiti-
zation by enhancing the MLCP activity in the smooth muscle (46).
The function of hHS-M21 appears opposite to that of telokin,
but the production of hHS-M21 from the MYPT2 gene is anal-
Fig. 5. Schematic representation of recombinant hHS-M21 proteins and their effects on \([\text{Ca}^{2+}]\), force relationship of \(\text{Ca}^{2+}\)-induced contraction. A, wild types and mutants of hHS-M21 proteins are schematically indicated along with their covering residues. Dotted and open box indicate leucine zipper motifs of hHS-M21 A and hHS-M21 B, respectively. B and C show the \([\text{Ca}^{2+}]\), force relation curves of contractions induced by increment of \(\text{Ca}^{2+}\) levels in absence (control, open circles) or presence of recombinant proteins, hHS-M21 A (closed circles), hHS-M21 B (open triangles), and hHS-M21 t-1 (closed squares). Force development was expressed as percentage of that obtained in the absence (control, open circles) or presence of hHS-M21 A and hHS-M21 B, respectively. B and C show the \([\text{Ca}^{2+}]\), force relation curves of contractions induced by increment of \(\text{Ca}^{2+}\) levels in absence (control, open circles) or presence of recombinant proteins, hHS-M21 A (closed circles), hHS-M21 B (open triangles), and hHS-M21 t-1 (closed squares). Force development was expressed as percentage of that obtained at 10 \(\mu\)M \(\text{Ca}^{2+}\). Data are represented by means \(\pm\) S.E. (n = 3–5). B, in 1% Triton X-permeabilized porcine renal artery. Amount of applied recombinant hHS-M21 proteins was 3 \(\mu\)M in each case. C, in 2.5% \(\beta\)-escin rat cardiac myocytes. Amount of applied hHS-M21 proteins was 1 \(\mu\)M in each case.

Table II

| Applied hHS-M21 protein | EC\textsubscript{50} (mean \(\pm\) S.E.) |
|------------------------|----------------------------------|
| 1                      | 434.8 \(\pm\) 17.4               |
| A                      | 86.9 \(\pm\) 2.0*                |
| B                      | 98.0 \(\pm\) 7.1*                |
| t-1                    | 118.4 \(\pm\) 2.4*               |
| t-2                    | 118.6 \(\pm\) 7.2*               |
| t-3                    | 428.2 \(\pm\) 29.1               |
| t-4                    | 408.9 \(\pm\) 33.2               |
| t-5                    | 373.5 \(\pm\) 34.1               |
| o-1                    | 300.7 \(\pm\) 21.6**              |
| o-2                    | 415.9 \(\pm\) 21.2               |

ogous to the situation of telokin produced by the MLCK gene. Although MYPT2 mRNA of 11.4 kbp was abundantly found in the heart and skeletal muscle (25), the MYPT2 protein (MYPT2-MBS) was reported to be expressed only in the heart and brain (25), in contrast to MYPT1-MBS, which is widely distributed among chicken or human tissues except for skeletal muscle (25, 47). In this study, we investigated the expression of MYPT2-MBS and hHS-M21 in various human tissues at the mRNA level by the RT-PCR analysis (Fig. 4). The amount of MYPT2 mRNA was slightly more abundant than that of MYPT1 mRNA in the cardiac and skeletal muscles, while the MYPT1 mRNA was much abundant as compared with MYPT2 mRNA in the brain and smooth muscle tissues (Fig. 4B). These findings were consistent with that the MYPT1-MBS was present in most tissues except for skeletal muscle, and that it appeared more abundant in the smooth and cardiac muscles than in the other tissues (47). Although both MBSs encoded by MYPT1 and MYPT2 increase the activity of MLCP, MYPT1-MBS was a more efficient activator than MYPT2-MBS, because MYPT2-MBS required about 10-fold higher concentration to achieve the same extent of activation as MYPT1-MBS for which maximum activation was found at approximately equimolar ratio of MYPT1-MBS and catalytic subunit (25). These observations are in good agreement with the present findings that MYPT2-MBS, and not MYPT2-MBS, was a main regulatory/target subunit bound by hHS-M21 to modulate the MLCP activity in the cardiac muscle, as in the smooth muscle.

The function of MYPT2-MBS is yet unclear. It was observed in this study that hHS-M21 proteins bound to the C-terminal one-third of MYPT2-MBS to a lesser extent (Fig. 6). This binding appears not to be background, because hHS-M21 proteins did not bind at all to the N-terminal two-thirds of MYPT2-MBS under the same conditions (Fig. 6A) and several truncated hHS-M21 mutants, t-1, t-2, and t-4, showed a weak binding to the C-terminal MYPT2-MBS (Fig. 6B). It also may be worth noting that a weak binding of the hHS-M21 t-4 protein to MYPT2-MBS was observed to a similar level as that to MYPT1-MBS. Although we could not demonstrate the \(\text{Ca}^{2+}\)-sensitizing effect of hHS-M21 t-4 in the smooth muscle (Table II), we cannot exclude the possibility that there might be other function(s) than the \(\text{Ca}^{2+}\)-sensitization of contraction, which is conferred by interaction between the C-terminal half of hHS-M21 and MYPT2-MBS, in the cardiac muscle. Further studies will be required to elucidate the function of MYPT2-MBS.

The C terminus of MYPT1-MBS was reported to contain binding sites for sm-M20 (12, 18, 19), Rho A (48), arachidonic acid (49), and acidic phospholipids (50). We have demonstrated
here that hHS-M21 interacts with the C-terminal one-third of MYPT1-MBS and increases the Ca\(^{2+}\) sensitivity of muscle contraction. There might be no evidence for the direct involvement of hHS-M21 in the Ca\(^{2+}\) sensitization effect. However, it was reported that the Ca\(^{2+}\)-calmodulin MLCK complex induced an increase in Ca\(^{2+}\) sensitivity in rat single skinned cardiac cells (51), and the movements of tension/pCa relationships were in similar extent to that observed in this study (Fig. 5C). It is therefore likely that the increase in the Ca\(^{2+}\) sensitivity by hHS-M21 is a reflection of the increased MLC phosphorylation. In addition, the binding domain to MYPT1-MBS and the main activating domain in Ca\(^{2+}\) sensitization were mapped to the N-terminal half region of the hHS-M21 in this study. It is suggested, then, that the exogenously applied recombinant hHS-M21 proteins may bind to endogenous MYPT1-MBS and exhibit the inhibitory action on the MLCP activity.

On the other hand, the N-terminal 56 residues of hHS-M21 (representative of hHS-M21 t-1) were sufficient for full activity of binding to the C-terminal one-third of MYPT1-MBS (Fig. 6), but they were not enough to confer full activity in increasing the Ca\(^{2+}\) sensitivity as demonstrated by hHS-M21 t-2 (residues 1–110) (Table II). However, neither binding activity nor Ca\(^{2+}\) sensitization effect was found with hHS-M21 o-2 that encompassed residues 57–110 (Fig. 6 and Table II). These results suggest that the main binding domain and the main active domain of hHS-M21 are overlapped considerably in the N-terminal 56 residues and that the main active domain is extended to C-terminal side of 57th residue but not exceeds the 110th residue. It is noteworthy that the main active domain of sm-M20 was mapped in the N-terminal half region (21). Because the C-terminal halves of hHS-M21 and sm-M20 have virtually identical amino acid sequences and no enhancing function of Ca\(^{2+}\) sensitivity, these observations suggest that the C-terminal halves of MLCP small subunits are dispensable for their functions in regulation of muscle contraction. In turn, these findings indicated that the N-terminal halves of hHS-M21 and sm-M20 exhibit their functions despite the low similarity in amino acid sequences. It will be interesting to determine which motifs in the N-terminal half of MLCP subunit would confer the function. Further investigations including site-directed mutagenesis of MLCP small subunit genes will be needed to demonstrate the functional motif(s).

The effect of hHS-M21 on the Ca\(^{2+}\) sensitivity was prominent in the smooth muscle as compared with in the cardiac muscle. The apparent difference in the efficiency of Ca\(^{2+}\) sensitization by hHS-M21 in these muscles may be due to the difference in the amount of recombinant proteins used in the assays. This possibility is unlikely because the increases in Ca\(^{2+}\) sensitivity with the different amounts of hHS-M21 were constant within a range from 1 to 10 nM in the assay with the porcine renal artery and within a range from 1 to 3 μM in the assay with rat cardiac myocytes (data not shown). The other possibilities for the differences are the species difference, porcine versus rat, in the assay system and the different expression or activity level of MYPT1-MBS between the smooth and cardiac muscles because the expression of MYPT1-MBS was a little more abundant in the smooth muscle than in the cardiac muscle. It also is possible that the contraction of tissue (porcine renal artery) can be measured more prominently than that of single cells (rat cardiac myocytes), because the contraction power from tissue is a summation of that from single cells. In support of the last possibility, the effect of MLCK on force development found in demembraned heart muscle strips (52, 53) was stronger than that in single-skinned cardiac cells (51).

In the cardiac muscle, it has been poorly understood about the role of MLC phosphorylation. However, a recent report has focused on the relevance of MLC phosphorylation system in the cardiac hypotrophy (27), and our observations highlight the role of MLCP system in the cardiac muscle contraction via identification and functional analysis of the heart-specific MLCP subunit. This is the first report demonstrating that HHS-M21, a heart-specific MLCP small subunit, plays a role in the regulation of Ca\(^{2+}\)-dependent contraction in the cardiac muscle via binding to C-terminal one-third of MYPT1-MBS.

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Identification, Characterization, and Functional Analysis of Heart-specific Myosin Light Chain Phosphatase Small Subunit
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