

**ENZYME CATALYSIS AND  
REGULATION:**

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## Differential Regulation of Alternatively Spliced Endothelial Cell Myosin Light Chain Kinase Isoforms by p60<sup>Src</sup>\*

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The Ca<sup>2+</sup>/calmodulin-dependent endothelial cell myosin light chain kinase (MLCK) triggers actomyosin contraction essential for vascular barrier regulation and leukocyte diapedesis. Two high molecular weight MLCK splice variants, EC MLCK-1 and EC MLCK-2 (210–214 kDa), in human endothelium are identical except for a deleted single exon in MLCK-2 encoding a 69-amino acid stretch (amino acids 436–505) that contains potentially important consensus sites for phosphorylation by p60<sup>Src</sup> kinase (Lazar, V., and Garcia, J. G. (1999) *Genomics* 57, 256–267). We have now found that both recombinant EC MLCK splice variants exhibit comparable enzymatic activities but a 2-fold reduction of  $V_{max}$ , and a 2-fold increase in  $K_{0.5\text{ CaM}}$  when compared with the SM MLCK isoform, whereas  $K_m$  was similar in the three isoforms. However, only EC MLCK-1 is readily phosphorylated by purified p60<sup>Src</sup> *in vitro*, resulting in a 2- to 3-fold increase in EC MLCK-1 enzymatic activity (compared with EC MLCK-2 and SM MLCK). This increased activity of phospho-MLCK-1 was observed over a broad range of submaximal [Ca<sup>2+</sup>] levels with comparable EC<sub>50</sub> [Ca<sup>2+</sup>] for both phosphorylated and unphosphorylated EC MLCK-1. The sites of tyrosine phosphorylation catalyzed by p60<sup>Src</sup> are Tyr<sup>464</sup> and Tyr<sup>471</sup> within the 69-residue stretch deleted in the MLCK-2 splice variant. These results demonstrate for the first time that p60<sup>Src</sup>-mediated tyrosine phosphorylation represents an important mechanism for splice variant-specific regulation of non-muscle MLCK and vascular cell function.

The family of myosin light chain kinases (MLCK)<sup>1</sup> expressed in vertebrates are Ca<sup>2+</sup>/calmodulin-regulated enzymes that catalyze the transfer of phosphate from Mg<sup>2+</sup>-ATP to a serine

residue (Ser<sup>19</sup>) of regulatory myosin light chain (MLC<sub>20</sub>) (1, 2). Members of this MLCK family share structural similarity, with a catalytic core that binds Mg<sup>2+</sup>-ATP and MLC<sub>20</sub> and a regulatory segment involved in Ca<sup>2+</sup>/calmodulin-dependent activation. Comparisons of primary sequences deduced from cDNA clones demonstrate that skeletal and cardiac muscle MLCK isoforms represent gene products that differ from the gene encoding the smooth muscle and nonmuscle MLCK isoforms localized on human chromosome 3 (3–6). Furthermore, the physiological role of the gene-specific MLCK isoforms differs significantly in the regulation of actomyosin contraction. In skeletal and cardiac muscles, Ca<sup>2+</sup> binds to the regulatory thin filament protein complex containing troponin and tropomyosin and thus allows actin to activate sarcomeric myosin Mg<sup>2+</sup>-ATPase. Although MLCK does not initiate muscle contraction in these tissues, MLCK-mediated phosphorylation of MLC<sub>20</sub> may potentiate the rate and extent of force development (7, 8). In contrast, Ser<sup>19</sup> phosphorylation of MLC<sub>20</sub> by Ca<sup>2+</sup>/calmodulin-dependent enzyme MLCK is essential for the initiation of nonmuscle and smooth muscle contraction (1, 3, 9–11). In specific nonmuscle tissues, such as the vascular endothelium, this kinase is known to be involved in endothelial cell migration, cell retraction (12), endothelial cell barrier regulation (13), transendothelial migration of neutrophils (14, 15), and possibly apoptosis (16). The MLCK isoform abundantly expressed in smooth muscle (SM MLCK) generally exists as a 130- to 150-kDa protein that has been well characterized (for review see Refs. 3 and 11). However, Western blot screening of a variety of embryonic and adult smooth muscle and nonmuscle tissues revealed expression of a high molecular weight MLCK variant with electrophoretic mobility in the range of 208–214 kDa (17–19). Garcia and colleagues (20) subsequently sequenced the high molecular weight MLCK isoform cloned from a human endothelial cell cDNA library, revealing an open reading frame, which encodes a protein of 1914 amino acids. Both the low molecular mass (130–150 kDa) and high molecular mass (208–214 kDa) MLCK isoforms share essentially identical actin binding, MLC binding, catalytic, and Ca<sup>2+</sup>/CaM-regulatory domains. The extreme C-terminal kinase-related protein (KRP) domain, which binds myosin, is contained within both EC MLCK and SM MLCK but can be also expressed as an independent protein capable of stabilizing myofilaments *in vitro* (3, 21–23). The C-terminal half of the endothelial MLCK isoform (residues 923–1914) exhibits 99.8% homology to the human low molecular weight MLCK from hippocampus and substantial homology to published SM MLCK sequences from rabbit (94% homology), bovine (95% homology), and chicken (85% homology) (5, 6, 24, 25). However, the exact biological function of the 922-amino acid N-terminal portion, which is unique to the high

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<sup>1</sup> The abbreviations used are: MLCK, myosin light chain kinase; MLC<sub>20</sub>, regulatory myosin light chain; SM, smooth muscle; EC, endothelial cell; HUVEC, human umbilical vein endothelial cells; RT-PCR, reverse-transcriptase-polymerase chain reaction; kb, kilobase(s); bp, base pair(s); Ni-NTA, nickel-nitrilotriacetic acid; MOPS, 4-morpholinepropanesulfonic acid; MALDI TOF, matrix-assisted laser desorption time of flight; KRP, kinase-related protein; CaM, calmodulin.

molecular weight MLCK isoform, is completely unknown. We have previously found that increased levels of endothelial cell protein tyrosine phosphorylation evoked by thrombin or diperoxovanadate are tightly linked to increased MLCK<sub>20</sub> phosphorylation, activation of actomyosin contraction, and a dramatic decrease in endothelial cell barrier function (26, 27). Further studies demonstrated that the increased kinase activity in MLCK immunoprecipitates strongly correlated with increased EC MLCK phosphorylation on tyrosine residues (26). Endothelial cell MLCK was found to be stably associated with p60<sup>Src</sup> kinase after stimulation (26), consistent with potential direct regulation of endothelial MLCK activity by tyrosine phosphorylation. More recently, detailed analysis of MLCK transcripts expressed in human endothelial cells revealed several splice variants of the EC MLCK isoform with predominant expression of the full-length isoform (MLCK-1) and a variant, which is identical to MLCK-1 except for a deleted 69-residue stretch (amino acid residues 437–505) encoded by a single exon (MLCK-2) (28). Within this deleted 69-amino acid stretch is an SH2-binding domain and consensus sites for phosphorylation by the Src family kinases (Tyr<sup>464</sup>, Tyr<sup>485</sup>). To better understand the role and significance of tyrosine phosphorylation and the function of the novel N terminus in EC MLCK regulation, we have expressed both EC MLCK-1 and EC MLCK-2 isoforms as well as smooth muscle MLCK in the baculovirus system and have characterized the biochemical properties of the purified recombinant proteins. Furthermore, we have assessed the phosphorylation of MLCK-1, MLCK-2, and SM MLCK by p60<sup>Src</sup> kinase *in vitro*, mapped the p60<sup>Src</sup>-phosphorylation sites to Tyr<sup>464</sup> and Tyr<sup>471</sup> of MLCK-1, and investigated the effects of MLCK-1 phosphorylation by p60<sup>Src</sup> kinase on the MLCK enzymatic activity and regulation by Ca<sup>2+</sup>/calmodulin. Our studies indicate the novel up-regulation of high molecular weight endothelial MLCK-1 isoform activity by p60<sup>Src</sup>-induced tyrosine phosphorylation and demonstrate isoform-specific endothelial cell MLCK regulation.

## MATERIALS AND METHODS

### Reagents

Chemicals used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. Restriction and modification enzymes were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL), Roche Molecular Biochemicals (Indianapolis, IN), Life Technologies (Gaithersburg, MD), and Promega (Madison, WI). Radioactive nucleotides, [ $\gamma$ -<sup>32</sup>P]ATP and [ $\gamma$ -<sup>33</sup>P]ATP, were obtained from PerkinElmer Life Sciences (Boston, MA). MLCK inhibitor (ML-7) and the p60<sup>Src</sup> inhibitor (PP2) were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Purified p60<sup>C-src</sup> kinase was obtained from Upstate Biotechnology (Lake Placid, NY). Sf9 and Hi5 insect cells (Invitrogen, Carlsbad, CA) were grown in serum-free Sf-900 II SFM media obtained from Life Technologies.

### Recombinant Donor Plasmids for Baculovirus Expression

**Smooth-muscle MLCK**—A rabbit uterine smooth muscle MLCK full-length cDNA in a pGEM vector (24) was a generous gift from Dr. Patricia Gallagher (Indiana University). The plasmid was cut at the *Eco*52I site, followed by blunt-ending with Klenow enzyme. The MLCK insert was then released by digesting plasmid with *Xba*I, separated from the vector by agarose gel electrophoresis, and purified using a Prep-A-Gene (Bio-Rad, Hercules, CA) kit. The smooth muscle MLCK cDNA was ligated with the pFastBac Hta baculovirus donor plasmid (Bac-To-Bac baculovirus expression system, Life Technologies), which had been digested with *Stu*I and *Xba*I enzymes.

**Nonmuscle MLCK-1 and MLCK-2**—MLCK-2 cDNA was obtained from human umbilical vein endothelial cells (HUVEC) by RT-PCR, amplified, and subcloned as described earlier (28), using sense primer 5'-ACT GAA TTC ACC ATG GGG GAT GTG AAG CTG and antisense primer 5'-GTC AGA ATT CTT GTT TCA CTC TTC TTC TTC C, both containing an *Eco*RI site. The EC MLCK-2 cDNA was inserted into pFastBac Hta vector at the *Eco*RI site. To generate MLCK-1 cDNA, an ~1.85-kb 5'-end fragment of HUVEC MLCK was amplified by RT-PCR

using a SuperScript preamplification system (Life Technologies). The first-strand cDNA was synthesized from HUVEC total RNA with oligo-dT primer. The PCR primers were as follows, sense: 5'-ACT GCG GCC GCA CCA TGG GGG ATG AGC TG (*Not*I restriction site followed by HUVEC MLCK-specific 5'-end sequence), and antisense: 5'-GTA CTC ACT CTT CCT GCT ACT C (~1.85-kb downstream HUVEC MLCK sequence). The same region (with a 207-bp deletion compared with EC MLCK-1) of the nonmuscle MLCK-2/pFastBac Hta plasmid was excised and replaced with this PCR-amplified fragment encoding the N-terminal portion of MLCK-1. Briefly, the PCR product was digested with *Not*I, blunt-ended, and cut by *Blp*I. The respective ~1.66-kb fragment from the nonmuscle MLCK-2/pFastBac Hta plasmid was excised with *Ehe*I and *Blp*I, and separated on agarose gel. The PCR-amplified 5'-end fragment of MLCK-1 and the 3'-end MLCK region previously subcloned into pFastBac Hta vector were ligated to create the recombinant donor plasmid of the whole EC MLCK-1 coding region reported previously (20). All three constructs (SM MLCK, EC MLCK-1, and EC MLCK-2) were verified by restriction analysis, PCR, and complete sequencing.

**Recombinant Regulatory Myosin Light Chain**—A plasmid pET3-MLC encoding rabbit vascular smooth muscle regulatory MLC was generously provided by Dr. Patricia Gallagher (Indiana University) and amplified by PCR using primers with unique restriction sites for directional cloning, MLC-sense-*Eco*RI: 5'-TGC TTT GAA TTC ATG TCC AGC AAG CGG GCC AAA GCC AAG-3', and MLC-antisense-*Xba*I: 5'-AAG GAC TCT AGA CTA GTC GTG TTT ATC CTT GGC GCC ATG-3', and then ligated with the pFastBac Hta baculovirus donor plasmid (Bac-To-Bac baculovirus expression system), which had been digested with *Eco*RI and *Xba*I enzymes.

### Baculovirus Expression of MLC and MLCK Isoforms

Human endothelial cell MLCK-1 and MLCK-2, rabbit smooth muscle MLCK and MLC recombinant baculovirus stocks were prepared using the Bac-To-Bac baculovirus expression system (Life Technologies) according to manufacturer's instructions. The system uses site-specific transposition of foreign genes into a baculovirus shuttle vector, bacmid, propagated in *Escherichia coli*. Sf9 and Hi5 insect cell suspension cultures at  $2 \times 10^6$  cells/ml were infected with the respective viral stock at a multiplicity of infection range of 0.1–10, and after 1-h incubation, diluted 5-fold with fresh media and grown for 2–4 days at 28 °C with continuous shaking. The optimal conditions varied for the three different MLCK isoforms expressed (not shown). For large scale expression and purification, Sf9 cells were infected with baculovirus (multiplicity of infection = 1), and the cells producing SM MLC, rabbit SM MLCK, or human endothelial MLCK-1 and -2 were harvested. The recombinant MLCK and MLC proteins contained a histidine tag at their N-terminal region introduced during subcloning MLCK cDNAs into the pFastBac Hta baculovirus donor vector. Expression of the MLCK isoforms was confirmed by SDS-polyacrylamide gel electrophoresis (29) and Western blot (30) using MLCK-specific D119 antiserum (31) or commercial anti-His tag antibodies.

### Purification of Recombinant Proteins

For the isolation of the recombinant MLCK isoforms or MLC, the infected Sf9 cells were harvested by centrifugation at  $3000 \times g$  for 5 min and frozen at -80 °C. Frozen insect cells were lysed (1:5 w/v ratio) in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.5, 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) at 4 °C for 2 min. The lysate was centrifuged at  $10,000 \times g$  for 10 min, and the supernatant was loaded onto Ni-NTA resin (Qiagen, Santa Clarita, CA). After a wash step with buffer A (20 mM Tris-HCl, pH 8.5, 500 mM KCl, 5 mM 2-mercaptoethanol, 10% glycerol), the expressed MLCK isoforms were eluted with 100 mM imidazole, 20 mM Tris-HCl, pH 8.5, 100 mM KCl, 5 mM 2-mercaptoethanol, 10% glycerol. The protein concentration was determined by Bio-Rad protein assay. The yield of MLCK isoforms ranged from 1.5 to 4 mg of MLCK from a 10-g Sf9 cell pellet. The purified enzymes were aliquoted and stored at -80 °C.

### Verification and Characterization of MLCK Constructs

Human endothelial MLCK-1 and MLCK-2 splice variants as well as the rabbit smooth muscle MLCK isoform cDNAs were subcloned into baculovirus vector pFastBac Hta as described above. The alternatively spliced regions in MLCK-1 and MLCK-2 mRNAs were verified by PCR amplification (not shown) using primers located upstream and downstream of nucleotides 1428–1634 as well as by complete sequencing of the MLCK-1 and MLCK-2 cDNA inserts. Translation of completely sequenced cDNA inserts encoding EC MLCK splice variants revealed



four amino acid residue differences between the unique N-terminal sequence of the EC MLCK-1 and MLCK-2 (Phe<sup>629</sup>, Cys<sup>681</sup>, Gly<sup>714</sup>, and Leu<sup>806</sup>) and the previously reported EC MLCK cDNA sequence (GenBank<sup>®</sup> accession number U48959). These sequence differences, which may represent polymorphisms within the human *MLCK* gene, did not involve either the consensus sequence for potential tyrosine phosphorylation catalyzed by p60<sup>src</sup> (Tyr<sup>464</sup>, Tyr<sup>471</sup>, Tyr<sup>485</sup>), the putative SH2-binding sites (Tyr<sup>69</sup>, Tyr<sup>464</sup>), the SH3 domains (Pro<sup>314</sup>-Arg<sup>318</sup>, Arg<sup>373</sup>-Pro<sup>379</sup>) previously proposed for EC MLCK (26, 28), and did not affect MLCK enzymatic properties (shown below). Rigorous analysis of the cDNA sequences encoding the C terminus of EC MLCK common to both high and low molecular weight MLCK isoforms, revealed three variances (Phe<sup>925</sup>/Leu, Ala<sup>1179</sup>/Val, and Lys<sup>1233</sup>/Glu) in the baculovirus-expressed recombinant MLCK-1 and MLCK-2 isoforms that do not correspond to GenBank<sup>®</sup> accession number 48959 or to homologous regions of the reported human, rabbit, and bovine SM MLCK variants, respectively (6, 24, 25). Leu<sup>925</sup> resides within the putative actin-binding domain spanning residues 910–1036 of MLCK-1, whereas Val<sup>1179</sup> and Glu<sup>1233</sup> do not lie within functional domains described for smooth muscle MLCK (32).

#### Myosin Light Chain Phosphorylation Assays

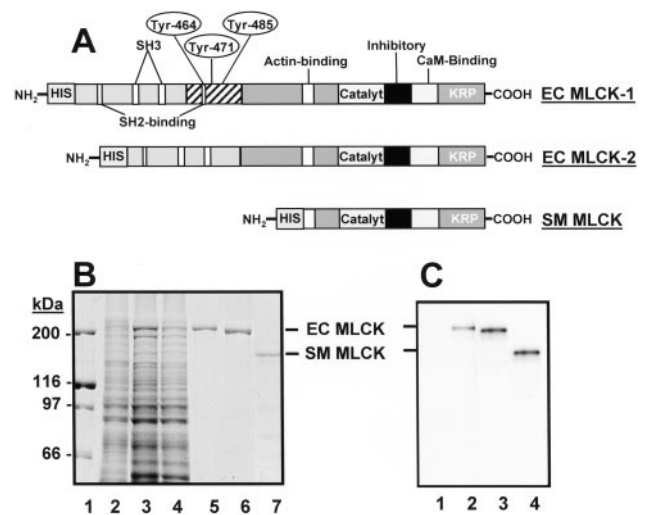
Baculovirus-expressed SM MLC was used as a substrate after His-tag excision by rTEV protease. In addition, several types of recombinant *Xenopus* regulatory MLC, including wild type MLC (Ser<sup>19</sup>Thr<sup>18</sup>) and Ser<sup>19</sup>Ala<sup>18</sup> and Ala<sup>19</sup>Thr<sup>18</sup> mutants expressed in *E. coli* (33) were used as substrates in studies of EC MLCK substrate specificity. The purified MLCKs were diluted in 50 mM MOPS, pH 7.4, 10 mM Mg<sup>2+</sup> acetate, 0.05% 2-mercaptoethanol containing 1 mg/ml bovine serum albumin to a  $1.25 \times 10^{-11}$  M final assay concentration. The MLCK activity was determined by measuring <sup>32</sup>P incorporation into the regulatory MLC used as substrate. The MLCK kinase assays were performed in 50 mM MOPS, pH 7.4, 10 mM Mg<sup>2+</sup> acetate, 0.025% 2-mercaptoethanol, in the presence 0.3 mM CaCl<sub>2</sub>, 10<sup>-6</sup> M calmodulin, 10<sup>-7</sup> M [ $\gamma$ -<sup>32</sup>P]ATP at 0.5 Ci/mmol specific activity, and 1.25–15  $\times 10^{-6}$  M myosin light chain at 22 °C as previously described (31). The concentration of free [Ca<sup>2+</sup>] in kinase reactions was calculated as described by Imai and Okeda (34) using the equation:  $p[Ca^{2+}] = 2 \text{ pH} - 7.28 + \log([EGTA]_{\text{added}}/[CaCl_2]_{\text{added}} - 1)$ .  $K_m$  and  $V_{\text{max}}$  values were determined from Lineweaver-Burk double-reciprocal plots using SigmaPlot software (SPSS Inc., Chicago, IL).

#### Phosphorylation of MLCK by p60<sup>src</sup> in Vitro

Purified MLCKs were dialyzed and brought to 0.1 mg/ml concentration using reaction buffer containing 25 mM Tris-HCl, pH 7.5, 20 mM KCl, 5 mM Mg<sup>2+</sup> acetate, 0.5 mM leupeptin. Phosphorylation of MLCK diluted in reaction buffer was started by adding 0.2 mM ATP, 10  $\mu$ Ci/ml [ $\gamma$ -<sup>32</sup>P]ATP and 75 units/ml recombinant p60<sup>src</sup> kinase (Upstate Biotechnology, Inc., Lake Placid, NY; final concentrations). Synthetic MLCK-1 peptides were used for p60<sup>src</sup> phosphorylation assays at 0.1 mg/ml final concentration. In certain experiments, a putative specific p60<sup>src</sup> inhibitor, PP-2 (Calbiochem-Novabiochem Corp., La Jolla, CA), was added to reaction tubes at 500 nM final concentration. The phosphorylation reaction was performed at 22 °C, and 10- $\mu$ l aliquots of reaction mixture were applied onto cellulose phosphate filters P81 (Whatman, UK) at specified periods of time. The filters were washed to remove unincorporated label, and specific incorporation of <sup>32</sup>P into MLCK was determined by scintillation counting. The p60<sup>src</sup> phosphorylation of the MLCK synthetic peptides as substrates was performed during 30 min at 22 °C under the same conditions. For scintillation counting, synthetic MLCK peptides were spotted onto nitrocellulose, and unbound radioactive label was washed out with solution containing 20% methanol and 2% disodium pyrophosphate. After completion of the phosphorylation reaction, phospho-MLCKs were aliquoted and stored at -80 °C until use in kinase assays.

#### Tryptic Cleavage and MALDI TOF Analysis of MLCK-1 Phosphopeptides

A 200- $\mu$ l aliquot of MLCK-1 (0.3 mg/ml) phosphorylated by p60<sup>src</sup> *in vitro* was partially digested by incubation with trypsin (1:100 w/w) for 5 min at room temperature in buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 5 mM MgCl<sub>2</sub>. The reaction was terminated by adding phenylmethylsulfonyl fluoride at 2 mM final concentration. The reaction mixture was lysed in SDS-sample buffer, peptides were separated on SDS-polyacrylamide gel electrophoresis, and phosphotyrosine peptides were identified by autoradiography and Western blot with anti-phosphotyrosine antibody. A portion of the polyacrylamide gel containing a



**FIG. 1. Baculovirus expression and purification of recombinant MLCK isoforms.** A, schematic representation of the domain organization of recombinant endothelial MLCK-1, MLCK-2, and smooth muscle MLCK expressed in baculovirus system. Depicted are the actin-binding domain, catalytic core, (catalyst), the regulatory segment containing the inhibitory and calmodulin-binding domains, and the kinase-related protein (KRP) domain. The unique N-terminal portion of the endothelial MLCK isoforms contains putative SH3- and SH2-binding domains. A 69-residue fragment (hatched region) containing a putative SH2-binding domain and the potential p60<sup>src</sup> phosphorylation sites (Tyr<sup>464</sup>, Tyr<sup>471</sup>, and Tyr<sup>485</sup>) is not expressed by either MLCK-2 or SM MLCK. The histidine tag (HIS) has been introduced at the C termini of recombinant MLCKs for purification purposes. B, purification of recombinant MLCK expressed in the baculovirus expression system using metal ion chromatography. The Coomassie Blue-stained gel demonstrates the results of MLCK purification using MLCK-1 as an example. Lane 1, molecular weight standards; lane 2, control cell lysate; lane 3, total lysate of MLCK-1-producing cells; lane 4, flowthrough fraction after Ni-NTA column chromatography; lane 5, purified MLCK-1 eluted from Ni-NTA resin with elution buffer containing 100 mM imidazole (see "Materials and Methods"). Lanes 6 and 7 represent the purified MLCK-2 and smooth muscle MLCK, respectively, after identical purification procedures. C, Western blot verification of recombinant MLCK isoforms using anti-MLCK antiserum D119. Lane 1 represents control Sf9 lysate; lanes 2–4 depict anti-MLCK immunoreactivity of purified recombinant MLCK-1, MLCK-2, and smooth muscle MLCK, respectively, after elution from the Ni-NTA column.

major 55-kDa MLCK-1 tryptic peptide, which incorporated <sup>32</sup>P and cross-reacted with anti-phosphotyrosine antibody, was excised and further processed. After 3  $\times$  30 min washes in distilled water, the gel piece was trimmed and the phosphopeptide incorporated into gel was subjected to destaining and complete trypsinolysis. Gel destaining required the addition of 100  $\mu$ l of 1:1 (v/v) acetonitrile:25 mM ammonium bicarbonate for ~30 min. The gel was then dried down completely using a Speed Vac concentrator (Savant), and further trypsinolysis was performed. The gel was incubated overnight at 37 °C with 1  $\mu$ l of trypsin solution (0.1  $\mu$ g/ $\mu$ l in 1% acetic acid) and 25  $\mu$ l of 25 mM ammonium bicarbonate, pH 7.8. After trypsinolysis, the MLCK peptides were eluted from the gel with a 1:1 (v/v) acetonitrile:water, 5% trifluoroacetic acid solution and concentrated to several microliters by Speed Vac. This peptide mixture was then analyzed by mass spectrometry. Mass spectra were acquired on a Kratos Axima CFR (Manchester, United Kingdom) time-of-flight mass spectrometer. Briefly, an aliquot of the peptide digest (0.3  $\mu$ l) was placed on the sample plate followed by the addition of 0.3  $\mu$ l of saturated ammonium sulfate and 0.3  $\mu$ l of matrix solution (saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid in 1:1 (v/v) ethanol:water). The mixture was air-dried (~10 min), and the sample plate was inserted into the mass spectrometer.

#### RESULTS

**Kinetic Characteristics of Recombinant Endothelial Cell MLCK Splice Variants**—The domain organization of the endothelial cell MLCK isoforms is schematically presented in Fig. 1A and demonstrates that the two endothelial isoforms: MLCK-1 and MLCK-2 are identical with the exception of the

TABLE I  
Kinetic properties and substrate specificity of recombinant MLCK-1, MLCK-2, and SM MLCK isoforms

Purified recombinant MLCK isoforms were used for *in vitro* kinase assay. Reaction mixtures were incubated with purified wild type MLC<sub>20</sub> as substrate, as described under "Materials and Methods."  $K_m$  and  $V_{max}$  were derived from double-reciprocal plots.  $K_{0.5 \text{ CaM}}$  was determined at  $5 \times 10^{-6}$  M smooth muscle MLC<sub>20</sub> final concentration and at saturating  $\text{Ca}^{2+}$  concentration. Kinase activity of recombinant MLCKs toward wild type (wt) and mutant MLC<sub>20</sub> is expressed as  $10^{-8}$  mol of phosphate incorporated per mg of MLCK per min. The concentrations of wild type and mutant MLC<sub>20</sub> were  $5 \times 10^{-6}$  M. The kinetic data represent the mean  $\pm$  S.D. of at least five separate experiments. The difference in  $K_{0.5 \text{ CaM}}$  and  $V_{max}$  between the EC MLCK and SM MLCK isoforms is statistically significant ( $p < 0.01$ ).

MLCK isoform	$V_{max}$	$K_{0.5 \text{ cam}}, 10^{-9} \text{ M}$	$K_m, 10^{-6} \text{ M}$	Kinase activity		
				MLC wt	MLC mutants	
				Thr <sup>18</sup> /Ser <sup>19</sup>	Thr <sup>18</sup> /Ala <sup>19</sup>	Ala <sup>18</sup> /Ala <sup>19</sup>
	$10^{-6} \text{ mol/mg/min}$			$10^{-9} \text{ mol/mg/min}$		
MLCK-1	$11.9 \pm 3.2$	0.49	$6.5 \pm 2.2$	$330 \pm 8$	$3.8 \pm 0.7$	$0.4 \pm 0.2$
MLCK-2	$10.9 \pm 1.8$	0.43	$7.2 \pm 2.8$	$361 \pm 8$	$3.7 \pm 1$	$0.6 \pm 0.5$
SM MLCK	$17.0 \pm 2.5$	0.21	$5.2 \pm 0.4$	$859 \pm 25$	$9.8 \pm 3.2$	$2.6 \pm 0.9$

deletion of a 69-residue stretch encoded by a single exon in MLCK-2 (28). The baculovirus-expressed and -purified MLCK isoforms (MLCK-1, MLCK-2, and SM MLCK) were analyzed by gel electrophoresis and revealed protein bands with expected sizes 214, 206, and 150 kDa, respectively (Fig. 1B), which reacted on the Western blot with MLCK-specific D119 anti-serum (Fig. 1C). After purification and rigorous sequencing analysis, the three recombinant MLCK isoforms were examined for their intrinsic enzymatic properties. Both EC MLCK-1 and EC MLCK-2 exhibited comparable  $V_{max}$  values ( $11.9 \pm 3.2$  and  $10.9 \pm 1.8 \mu\text{mol/min/mg}$ , respectively), which were slightly reduced compared with the rabbit SM MLCK isoform ( $17.0 \pm 2.5 \mu\text{mol/min/mg}$ ) (Table I). The  $K_m$  values reflecting substrate affinity were not significantly different among the three recombinant MLCK preparations, whereas the  $K_{0.5 \text{ calmodulin}}$  was higher for the EC MLCK isoforms (0.49 and 0.42 nM) compared with the rabbit SM MLCK isoform (0.21 nM) (Table I). Overall, the  $K_m$ ,  $V_{max}$ ,  $K_{0.5 \text{ calmodulin}}$  values for the baculovirus-expressed endothelial MLCK-1 and MLCK-2 splice variants are in good agreement with published values for MLCK activity derived from other tissues (24, 35–37). Furthermore, all three recombinant MLCK isoforms possess identical substrate specificity and preferentially phosphorylate Ser<sup>19</sup> and Thr<sup>18</sup> of regulatory MLC<sub>20</sub> (Table I), because the substitution of Ala for either Ser<sup>19</sup> alone or both Ser<sup>19</sup> and Thr<sup>18</sup> in the MLC mutants resulted in dramatic reduction in MLC<sub>20</sub> phosphorylation. These results are in complete agreement with the previously reported preferred sequential phosphorylation of Ser<sup>19</sup> followed by phosphorylation at the MLC second site (Thr<sup>18</sup>), which is phosphorylated more slowly than Ser<sup>19</sup> and requires relatively high concentrations of myosin light chain kinase (2).

**In Vitro Phosphorylation of MLCK Isoforms by p60<sup>Src</sup>**—Augmentation of tyrosine protein phosphorylation in endothelial cells *in vivo* correlates with increased phosphotyrosine content in MLCK immunoprecipitates, increased MLCK activity, and increased MLC phosphorylation (26, 38). More recently, EC MLCK has been shown to be stably associated with p60<sup>Src</sup> and a well recognized p60<sup>Src</sup> substrate, the actin-binding protein cortactin (26). To further characterize the role of tyrosine phosphorylation in EC MLCK regulation, the three recombinant MLCK isoforms were used as substrates for *in vitro* phosphorylation catalyzed by p60<sup>Src</sup>. EC MLCK-1 exhibited substantial time-dependent p60<sup>Src</sup>-catalyzed incorporation of radioactive phosphate (Fig. 2), whereas p60<sup>Src</sup>-mediated <sup>32</sup>P incorporation did not occur in either MLCK-2, the EC MLCK splice variant, lacking the 69-amino acid stretch containing the p60<sup>Src</sup> consensus site, nor in SM MLCK, which completely lacks the novel N terminus (Fig. 2). The <sup>32</sup>P incorporation into MLCK-1 was essentially abolished by the specific p60<sup>Src</sup> kinase inhibitor PP-2 (250 nM). However, all three MLCK isoforms exhibited low level of <sup>32</sup>P incorporation even in the absence of p60<sup>Src</sup>

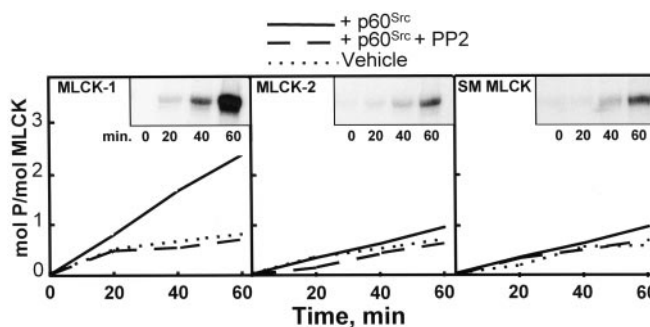
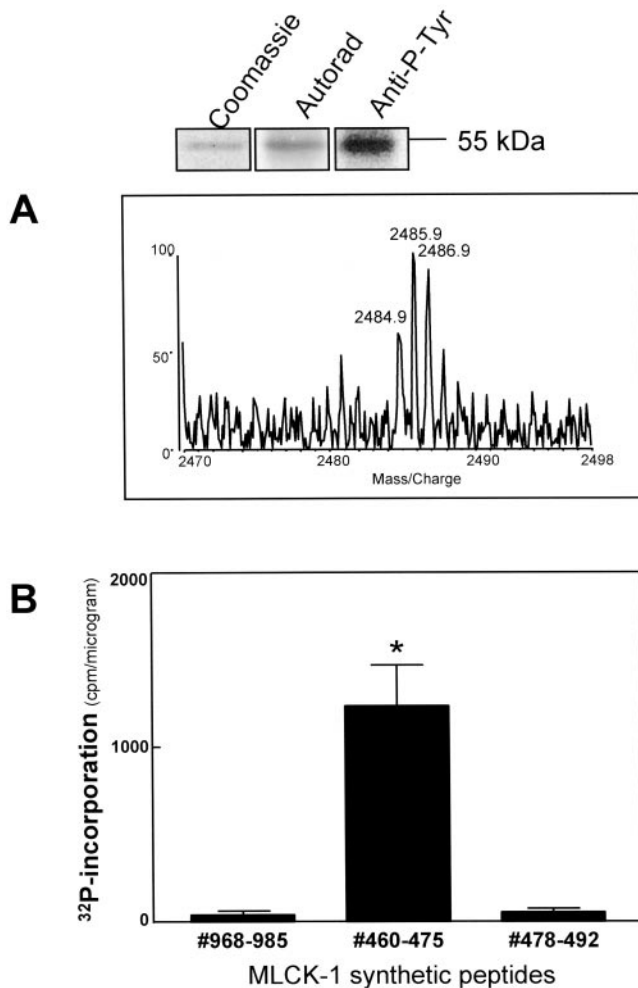


FIG. 2. Phosphorylation of MLCK-1, MLCK-2, and SM MLCK by p60<sup>Src</sup> *in vitro*. Purified recombinant MLCK-1 (left panel) and MLCK-2 (middle panel) and SM MLCK (right panel) expressed in the baculovirus system were phosphorylated using purified p60<sup>Src</sup> kinase as outlined under "Materials and Methods." The insets depict the representative autoradiograms of time-dependent <sup>32</sup>P incorporation into each MLCK isoform in the presence of [<sup>32</sup>P]ATP and p60<sup>Src</sup>. The graphs demonstrate the time dependence of <sup>32</sup>P incorporation into MLCK-1, MLCK-2, and SM MLCK incubated in the presence of p60<sup>Src</sup> alone (solid lines) or in combination with 500 nM PP-2, a specific inhibitor of p60<sup>Src</sup> (broken lines) or in the absence of p60<sup>Src</sup> (dotted lines). The results obtained in four independent experiments are expressed as the stoichiometry of <sup>32</sup>P incorporation into MLCK.

( $0.63 \pm 0.21$  mol of  $\text{PO}_4$ /mol of protein) consistent with the MLCK autophosphorylation previously described for smooth muscle MLCK (39, 40). This was confirmed by heat treatment of the MLCK preparations ( $70^\circ\text{C}$  for 5 min), which completely abolished incorporation of <sup>32</sup>P into MLCK in the absence of p60<sup>Src</sup> (data not shown). The preferential p60<sup>Src</sup>-catalyzed phosphorylation of MLCK-1 compared with other MLCK isoforms strongly suggested that the specific site of tyrosine phosphorylation of endothelial MLCK by p60<sup>Src</sup> resides within the 69-amino acid residue stretch in the N-terminal part of the MLCK molecule encoded by a single exon (28), which is not expressed in MLCK-2. However, the stoichiometry of phosphate incorporation into MLCK-1 catalyzed by p60<sup>Src</sup> ( $2.32 \pm 0.32$  mol of  $\text{PO}_4$ /mol of protein) strongly suggested the potential presence of a secondary tyrosine residue within MLCK-1, which may also be phosphorylated by p60<sup>Src</sup>.

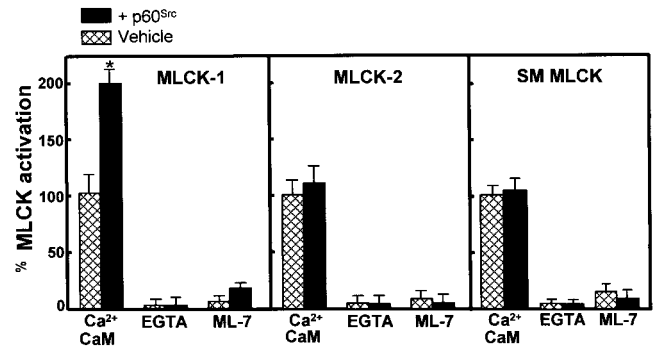
**Identification of p60<sup>Src</sup> Phosphorylation Sites within MLCK-1**—To search for tyrosine phosphorylation site(s) within MLCK-1, we applied mass spectroscopy analysis of tryptic fragments obtained from MLCK-1 phosphorylated by p60<sup>Src</sup>. As an initial step, a limited trypsinolysis of radiolabeled phospho-MLCK-1 was performed, and tyrosine phosphorylation site(s) mapped to the tryptic fragment with an approximate molecular weight of 55 kDa based on autoradiography data and Western blot analysis with anti-phosphotyrosine antibody (inset, Fig. 3A). Mass spectrometric analysis of MLCK-1 peptides obtained after complete tryptic digestion of a 55-kDa MLCK-1 fragment





**FIG. 3. Identification of the tyrosine phosphorylation sites within the N-terminal 55-kDa MLCK-1 tryptic fragment.** The  $p60^{src}$ -catalyzed phosphorylation of the 55-kDa tryptic MLCK-1 fragment was detected by autoradiography and immunoreactivity with anti-phosphotyrosine antibody (upper inset). After excision from the gel, the phosphoprotein was subjected to complete trypsinolysis as described under "Materials and Methods," and the peptide digest was analyzed using mass spectrometry. **A**, high mass spectrum resolution of the peak with an average  $m/z$  ratio 2486, which mathematically corresponds to tryptic fragment 457–476 of MLCK-1 with two incorporated phosphate groups. Shown are peaks with  $m/z$  ratios 2484.9, 2485.9, and 2486.9 corresponding to isotopic variants of the diphosphorylated MLCK-1 457–476 peptide. **B**, *in vitro*  $p60^{src}$ -catalyzed phosphorylation of synthetic peptides consisting of amino acids 968–985, 460–475, and 478–492 of MLCK-1. Each peptide was tested in kinase assay as outlined under "Materials and Methods." Each point represents the mean  $\pm$  S.E. of three experiments. \*, results are significant  $p < 0.005$ .

revealed a group of peptides corresponding to the N-terminal portion of MLCK-1 (Fig. 3A). Furthermore, we identified a single characteristic peak (Fig. 3A) not seen in the digests of MLCK-1 preincubated with ATP without  $p60^{src}$  (data not shown). This peak has  $m/z$  ratio 2486 corresponding to the theoretical mass of the tryptic peptide 457–476 of MLCK-1 ( $^{457}$ QEGSIEVYEDAGSHYLCLLK $^{476}$ ) with two incorporated phosphate groups (underlined). MALDI TOF analysis allowed isotopic resolution of the  $m/z$  2486 peak (isotopic variants with  $m/z$  ratios 2484.9, 2485.9, and 2486.9, respectively) further supporting mapping of the peak to the diphospho-(457–476)-MLCK-1 fragment. To further prove phosphorylation of sites Y $^{464}$  and Y $^{471}$  by  $p60^{src}$ , we used synthetic peptides  $^{460}$ SIEVYEDAGSHYLCLL $^{475}$  and  $^{478}$ RTRDSGTYSCTASNA $^{492}$  corresponding to amino acid residues 460–475 and 478–492 of full-length MLCK-1 protein, respectively, for *in vitro*  $p60^{src}$



**FIG. 4. Effect of phosphorylation of endothelial MLCK by  $p60^{src}$  on MLCK activity *in vitro*.** **A**, kinase activity of MLCK-1 after 60-min incubation with either  $p60^{src}$  (solid bars) or vehicle (cross-hatched bars). The MLC kinase assay utilized recombinant rabbit smooth muscle MLCs as substrate as described under "Materials and Methods." **B** and **C** are similar to **A** with the exception that MLCK-2 or SM MLCK were used in each reaction mixture. MLCK-2 and SM MLCK incubated with (solid bars) or without (open bars)  $p60^{src}$  under identical conditions were also tested for kinase activity. Removal of Ca $^{2+}$  from the kinase reaction mixture with EGTA or the addition of specific MLCK inhibitor ML-7 ( $5 \times 10^{-6}$  M) abolishes MLCK-1, MLCK-2, and SM MLCK activity. MLCK-catalyzed  $^{32}$ P incorporation into myosin light chains in the presence of Ca $^{2+}$  and calmodulin in the reaction mixture as described under "Materials and Methods" was taken as 100% MLCK activation. Each point represents the mean  $\pm$  S.E. of at least three experiments. \*, results are significant  $p < 0.005$ .

phosphorylation assay (Fig. 3B). Only the peptide containing Y $^{464}$  and Y $^{471}$  was readily phosphorylated in the presence of  $p60^{src}$ , whereas another candidate peptide containing the potential  $p60^{src}$  consensus phosphorylation site, Y $^{485}$ , as well as irrelevant peptide PEKVPKPPKATPDFRSVL (residues 968–985 of MLCK-1), which lacks tyrosine residues, were not phosphorylated. The relatively low stoichiometry of  $p60^{src}$ -mediated phosphate incorporation into peptide 460–475 ( $\sim 0.015$  mol of PO $_4$ /mol of peptide, 10-min reaction) suggests that other MLCK-1 epitope(s) in proximity to amino acid residues 460–475 may be required for the optimal  $p60^{src}$  activity. As recently demonstrated, the interaction of the  $p60^{src}$  SH3 domain with the ligand sequence of the substrate is important for activation of the catalytic domain and autophosphorylation (41), and the addition of an SH3 domain ligand to a substrate peptide increases its phosphorylation 10-fold via lowering of the  $K_m$  value of the substrate and kinase activation (42). Thus, putative SH3- and SH2-binding domains present in MLCK-1 N-terminal portion (Fig. 1A) may be important for  $p60^{src}$ -catalyzed MLCK-1 phosphorylation, and further studies are underway to address this question. Finally, mass spectrometry analysis of the fragments obtained after cyanogen bromide cleavage of the MLCK-1 suggested that the two phosphate groups are contained within the Lys $^{1721}$ -Met $^{1761}$  EC MLCK fragment (data not shown) consistent with the presence of the EC MLCK-1 autophosphorylation sites Thr $^{1748}$  and Ser $^{1760}$ , which are homologous to the previously described SM MLCK autophosphorylation sites Thr $^{803}$  and Ser $^{815}$  (39).

**Differential Activation of MLCK-1 and MLCK-2 by  $p60^{src}$ -mediated Phosphorylation**—To explore whether phosphorylation by  $p60^{src}$  alters endothelial MLCK-1 enzymatic properties, MLCK-1 samples were preincubated with either ATP and  $p60^{src}$  ("phospho-MLCK") or ATP alone ("dephospho-EC MLCK"), followed by assessment of *in vitro* kinase activity. Phosphorylation of EC MLCK-1 by  $p60^{src}$  increased EC MLCK-1 kinase activity 2-fold (Fig. 4), whereas the enzymatic activities of EC MLCK-2 and SM MLCK were not affected by  $p60^{src}$  and were comparable to that measured for EC MLCK-1 in the absence of  $p60^{src}$ . These results are again consistent with the inability of  $p60^{src}$  to phosphorylate EC MLCK-2 (as shown

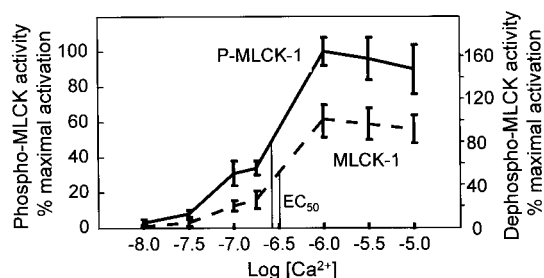


FIG. 5. Effect of  $p60^{src}$ -mediated MLCK-1 phosphorylation on  $Ca^{2+}$ -dependent MLCK-1 activation. The enzymatic activities of  $p60^{src}$ -treated MLCK-1 (solid line) or MLCK-1 incubated with vehicle and referred as dephospho-MLCK-1 (broken line) were measured in the presence of  $5 \times 10^{-7}$  M calmodulin over a range of  $Ca^{2+}$  concentrations ( $10^{-8}$  to  $10^{-5}$  M) as described under "Materials and Methods." The maximal activity of phospho-MLCK-1 observed in the presence of  $10^{-6}$  M  $[Ca^{2+}]$  was taken as 100%. Maximal activation of dephospho-MLCK-1 (incubated at  $10^{-6}$  M  $[Ca^{2+}]$ ) represents 55% of phospho-MLCK-1 activity. Each point represents the mean  $\pm$  S.E. of at least three experiments. \*, results are significant  $p < 0.005$ .  $EC_{50}$  values for  $Ca^{2+}$  dependence of dephospho- and phospho-MLCK-1 activation are outlined by vertical lines.

in Fig. 2) and indicate a significant enhancement of MLCK-1 kinase activity by  $p60^{src}$ -mediated phosphorylation. Enzymatic activity of all three isoforms was  $Ca^{2+}$ /CaM-dependent, because chelation of free  $Ca^{2+}$  with 2 mM EGTA (Fig. 4) or removal of calmodulin from the kinase reaction mixture (data not shown) completely abolished MLC phosphorylation catalyzed by either phospho- or dephospho-MLCK-1 preparations by MLCK-2 and SM MLCK. An inhibitor of smooth muscle MLCK activity, ML-7 ( $5 \times 10^{-6}$  M) also abolished the enzymatic activity of EC MLCK-1 (phospho- and dephospho-), EC MLCK-2, and SM MLCK (Fig. 4). Finally, phosphorylation of MLCK-1 by  $p60^{src}$  did not alter  $Ca^{2+}$ /CaM-dependent regulation, because the values for half-maximal activation of phospho- and dephospho-MLCK-1 determined over a range of free  $Ca^{2+}$  concentrations ( $10^{-8}$  to  $10^{-5}$  M) were comparable ( $pCa$  6.56 versus  $pCa$  6.50, respectively) despite an increase of  $\sim 2$ -fold in enzymatic activity toward  $MLC_{20}$  in the phospho-MLCK-1 preparation (Fig. 5). These data suggest that, although similar  $Ca^{2+}$  concentrations are required for MLCK-1 activity, tyrosine phosphorylation promotes increased MLC phosphorylation at lower  $Ca^{2+}$  concentrations within the cells.

#### DISCUSSION

In contrast to smooth muscle, only the high molecular weight MLCK isoform (208–214 kDa) is expressed in endothelium (18–20, 22). Molecular cloning of MLCK from human endothelial cells (20) revealed a high molecular weight MLCK variant containing a unique 922-residue N-terminal domain not expressed in the low molecular weight MLCK isoform, which is abundantly expressed in smooth muscle. Comparison of the cDNA encoding human high and low molecular weight MLCKs, when combined with results of chromosome mapping of human *MLCK* to single locus with chromosomal localization to 3qcen-q21 (6), suggests that mammalian *MLCK* genomic organization is highly similar to the "gene within a gene" organization of the avian smooth muscle/nonmuscle *MLCK* gene expressing two size class MLCK variants and one nonkinase protein (KRP), which are encoded by exons 1–31, 15–31, and 29A–31, respectively (17, 43). The complexity of the human *MLCK* genomic organization was recently further emphasized by the detection of five splice variants of high molecular weight MLCK in non-muscle and smooth muscle tissues using RT-PCR approaches (28). These data, which elucidated the considerable expression of EC MLCK-2, were strongly consistent with the potential functional diversity of the expressed smooth muscle and non-

muscle MLCK proteins. Among endothelial cell MLCK splice variants, the MLCK-1 and MLCK-2 appear to be preferentially expressed (28), although all five have been identified in tissues. Using purified recombinant MLCK-1 and MLCK-2 expressed in a baculovirus system, we have now characterized for the first time the kinetic parameters of the high molecular weight MLCK isoforms from human endothelial cells. Comparisons of the  $V_{max}$  and  $K_m$  of these high molecular weight isoforms to recombinant rabbit uterine smooth muscle MLCK reveal very similar enzymatic properties of the three MLCK isoforms. However, a 2-fold increase in  $K_{0.5}$  calmodulin observed in endothelial MLCK splice variants, may suggest a lower sensitivity of the intracellular EC MLCK for regulation by  $Ca^{2+}$ /calmodulin as compared with SM MLCK.

A number of protein kinases, including cAMP-dependent protein kinase A, protein kinase C,  $Ca^{2+}$ /CaM-dependent protein kinase II, and p21-activated kinase have been demonstrated to phosphorylate the smooth muscle MLCK isoform *in vitro* and *in vivo* (44–47). Serine/threonine phosphorylation within MLCK calmodulin-binding domain results in a 10-fold increase in  $K_{CaM}$  reflecting a 3.5-fold decrease in the association rate and a 6-fold increase in the dissociation rate between MLCK and  $Ca^{2+}$ /CaM (3, 45, 46, 48, 49) and thus reduced MLCK enzymatic activity. In turn, phosphorylation by p21-activated kinase decreases MLCK-1 catalytic activity by  $\sim 50\%$  via decrease in maximum velocity ( $V_{max}$ ) without affecting  $K_{CaM}$  (47). In addition, Thr<sup>803</sup>, Ser<sup>815</sup>, and Ser<sup>823</sup> of the smooth muscle MLCK isoform undergo autophosphorylation *in vitro* also resulting in decreased MLCK affinity to  $Ca^{2+}$ /calmodulin (39).

In contrast to serine/threonine phosphorylation of SM MLCK and EC MLCK, which attenuates MLCK activity (3, 20, 31, 46, 48), information is limited regarding phosphorylation sites within the MLCK molecule, which serve to enhance its enzymatic activity. Phosphorylation of smooth muscle MLCK by mitogen-activated protein kinase *in vitro* has been reported to stimulate smooth muscle MLCK activity (50), although we have not yet found mitogen-activated protein kinase to affect EC MLCK activity in this manner. However, recent studies have defined the involvement of tyrosine phosphorylation in EC MLCK regulation (26, 27, 51). Augmentation of protein tyrosine phosphorylation increased  $MLC_{20}$  phosphorylation and cell contraction in endothelial cells, which strongly correlated with an increase in MLCK phosphotyrosine content, enhanced MLCK enzymatic activity, and the stable association of EC MLCK with activated  $p60^{src}$  (26). Our present results appear to be consistent with the hypothesized novel role of the unique N terminus in EC MLCK regulation via tyrosine phosphorylation. We now demonstrate for the first time the *in vitro* phosphorylation of the full-length EC MLCK-1 by  $p60^{src}$  kinase on Tyr<sup>464</sup> and Tyr<sup>471</sup>, post-translational modifications not observed in the EC MLCK-2 splice variant lacking the 69-residue stretch (amino acids 436–505) in the N terminus, which is encoded by a single exon deleted in the EC MLCK-2 isoform (28). Our future studies using site-directed mutagenesis approach are aimed at the determination of the sequence of phosphorylation events and the role of each tyrosine phosphorylation site in the regulation of MLCK-1.

In summary, we have characterized the kinetic properties of endothelial MLCK splice variants and demonstrated a novel mechanism of MLCK-1 regulation by  $p60^{src}$  phosphorylation. The phosphorylation sites (Tyr<sup>464</sup> and Tyr<sup>471</sup>) are located within unique N-terminal domain (436–505) of endothelial MLCK-1 isoform not expressed in smooth muscle MLCK or in the alternatively spliced endothelial isoform MLCK-2. Consistent with this finding, only MLCK-1 activity is regulated by

p60<sup>src</sup>-catalyzed phosphorylation. These data demonstrate the importance of the novel N-terminal domain in the specific regulation of the MLCK isoform present in nonmuscle cells. As we have previously demonstrated, the tyrosine phosphorylation of EC MLCK increases its association with both p60<sup>src</sup> kinase, as well as with the actin-binding protein and the p60<sup>src</sup> substrate cortactin (26), we speculate that MLCK-1 tyrosine phosphorylation may be involved in contractile complex scaffolding and contribute to Ca<sup>2+</sup> sensitization of the endothelial contractile apparatus. Based on our data, we speculate that p60<sup>src</sup>-catalyzed tyrosine phosphorylation contributes to the local and selective activation of endothelial cell MLCK-1 under submaximal Ca<sup>2+</sup> concentrations providing a mechanism that may tightly orchestrate critical cytoskeletal rearrangements and ultimately the cellular contraction, which is critical for endothelial cell-dependent biological processes, such as vascular barrier regulation, transendothelial leukocyte diapedesis, and angiogenesis.

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