

# Characterization of the Protein Phosphatase 1 Catalytic Subunit in Endothelium: Involvement in Contractile Responses

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**Abstract** We have previously demonstrated the direct involvement of a type 1 Ser/Thr phosphatase (PPase 1) in endothelial cell (EC) barrier regulation [Am. J. Physiol. 269:L99–L108, 1995]. To further extend this observation, we microinjected either the Ser/Thr PPase inhibitor, calyculin, or the PPase 1 inhibitory protein, I-2 into bovine pulmonary artery EC and demonstrated both an increase in F-actin stress fibers and a shift from a regular polygonal shape to a spindle shape with gaps apparent at the cell borders. Northern blot analysis with specific cDNA probes revealed the presence of three major PPase 1 catalytic subunit (CS1) isoforms ( $\alpha$ ,  $\delta$ , and  $\gamma$ ) in human and bovine EC. To characterize the myosin-associated EC CS1 isoform, myosin-enriched bovine EC fraction was screened with anti-CS1 $\alpha$  and anti-CS1 $\delta$  antibodies. The anti-CS1 $\delta$  antiserum, but not anti-CS1 $\alpha$  antiserum cross reacts with the CS1 isoform present in myosin-enriched fraction and CS1 $\delta$  was found in stable association with EC myosin/myosin light chain kinase (MLCK) complex in MLCK immunoprecipitates under nondenaturing conditions. Consistent with these data, overexpression of CS1 $\delta$ -GFP construct in bovine endothelium followed by immunoprecipitation of CS1 with anti-GFP antibody revealed the stable association of CS1 $\delta$  with actomyosin complex. Finally, screening of a human EC oligo(dT)-primed cDNA library with a probe encoding a rat CS1 $\delta$  cDNA segment yielding several positive clones that encoded the entire CS1 $\delta$  open reading frame and partially noncoding regions. Sequence analysis determined a high homology ( $\approx 99\%$ ) with human CS1 $\delta$  derived from a teratocarcinoma cell line. Together, these data suggest that CS1 $\delta$  is the major of PPase 1 isoform specifically associated with EC actomyosin complex and which participates in EC barrier regulation. J. Cell. Biochem. 79:113–125, 2000. © 2000 Wiley-Liss, Inc.

**Key words:** microinjection; inhibitor-2; CS1 isoforms in endothelium

## INTRODUCTION

Vascular endothelial cell (EC) monolayers serve as a semi-selective barrier to fluid and sol-

ute flux across the blood vessel wall. Increased endothelial permeability is a prominent feature of acute inflammatory lung syndromes and is the result of intercellular gap formation evoked by bioactive agents such as the coagulation protease thrombin [Garcia et al., 1986, 1995]. We and others [Garcia et al., 1995; Goeckeler and Wysolmerski, 1995] have recently shown that actin-myosin interaction is a key event in the control of EC contractility and barrier function, and is regulated via reversible phosphorylation of the regulatory myosin light chain (MLC). For example, EC activation by thrombin induces MLC phosphorylation, actin redistribution, and filament formation, which is expedient to gap formation and barrier dysfunction [Garcia and Schaphorst, 1995]. MLC is phosphorylated by

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myosin light chain kinase (MLCK) in  $\text{Ca}^{2+}$ /calmodulin (CaM)-dependent fashion [Adelstein and Klee, 1981; Sellers et al., 1981]. We have recently cloned and studied the expression and regulation of a new, large molecular weight isoform of MLCK (214 kDa) in cultured endothelium that is unique and distinct from its smooth muscle (SM) MLCK counterpart (130–150 kDa) [Garcia et al., 1997; Verin et al., 1998A, B]. However, the actual phosphorylation state of MLC depends on not only MLCK activity, but the equilibrium between MLCK and myosin-associated protein phosphatase (PPase) activities as well [for review, see De Lanerolle and Paul, 1991; Somlyo and Somlyo, 1994]. Each of the four main classes of Ser/Thr PPases are able to dephosphorylate MLC *in vitro* [Ingebritsen and Cohen, 1983; Pato et al., 1983; Stewart et al., 1983] and include a type 1 protein phosphatase (PPase 1) and three type 2 enzymes; PPase 2A, PPase 2B, and PPase 2C, which can be distinguished on the basis of substrate specificity, divalent cation requirements, and their susceptibility to selective PPase inhibitors [for review, see Cohen, 1989; Shenolikar and Nairn, 1991]. It was recently demonstrated that PPase 1 is responsible for dephosphorylation of myosin in smooth muscle [Alessi et al., 1992]. The smooth muscle PPase 1 that is bound to myofibrils consists of several regulatory subunits 130 kDa (M130), 20 kDa (M20), and 37 kDa catalytic subunit (CS1), which was found to represent the  $\delta$  isoform (CS1 $\delta$ ) [Chen et al., 1994A; Okubo et al., 1994; Shimizu et al., 1994; Shirazi et al., 1994]. We have recently demonstrated direct involvement of PPase 1 in the regulation of EC gap formation and barrier function in pulmonary artery endothelium by studying the effect of semiselective protein PPase inhibitors on the extent of MLC phosphorylation, myosin-associated PPase activity, and EC monolayer permeability [Verin et al., 1995]. Consistent with these data, differential inhibition of PPases 1 and 2A by okadaic acid suggested that PPase 1 is involved in the regulation of pulmonary microvascular EC barrier function [Diwan et al., 1997]. Additional immunocytochemical data and inhibitory analysis indicated PPase 1 involvement in sustaining the normal cytoskeletal structure in umbilical vein endothelium [Shinoki et al., 1995]. Furthermore, recent studies have demonstrated that EC activation by thrombin leads to inhibition of myosin-associated PPase 1 activity [Shasby et al., 1997; Verin et al., 1998] possibly due to thrombin-induced dissocia-

tion of CS1 from the functional actomyosin protein complex [Verin et al., 1998]. Despite this compelling evidence for PPase1 participation in physiologic responses, information is limited about structural and functional characteristics of EC PPase 1. In the present work, we have further characterized PPase 1 in endothelium and demonstrate the presence of  $\alpha$ ,  $\gamma$ , and  $\delta$  isoforms in EC and the CS1 $\delta$  is the isoform associated with EC myosin-based contractile protein complex.

## MATERIALS AND METHODS

### Materials

Nonmuscle antimyosin antibody were purchased from Berkeley Antibody Co. (Richmond, CA). Monoclonal anti-GFP antibody were obtained from Zymed Laboratories (San Francisco, CA). Anti-MLCK (D119) antibody were kindly provided by Dr. Patricia Gallagher (Indianapolis, IN). Antiserum against CS1 $\delta$ , purified recombinant CS1 $\alpha$  and  $\delta$  isoforms and recombinant PPase 1 inhibitory protein (I-2) were kindly provided by Dr. Anna DePaoli-Roach. (Indianapolis, IN). Anti-CS1 $\alpha$  antibody were purchased from Upstate Biotechnology (Lake Placid, NY). A dT-primed human umbilical vein endothelial cell cDNA library was a generous gift from Dr. David Ginsburg (Ann Arbor, MI). Human uterus total RNA and pEGFP-C1 vector were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Nitrocellulose filters and kaleidoscope prestained molecular weight standards for SDS-PAGE were obtained from Bio-Rad Laboratories (Hercules, CA). Other reagents were reagent grade from Sigma and Bio-Rad Co.

### Bovine Pulmonary Artery Endothelial Cell (BPAEC) Culture

BPAEC were obtained frozen at 16 passages from American Type Tissue Culture Collection (Rockville, MD) (culture line, CCL 209) and were utilized at passage 19–24 as previously described [Stasek et al., 1992]. Cells were cultured in M-199 media (Gibco) supplemented with 20% (v/v) colostrum-free bovine serum (Irvine Scientific, Santa Ana, CA), 15  $\mu\text{g}/\text{ml}$  endothelial cell growth supplement (Collaborative Research, Bedford, MA), 1% antibiotic and antimycotic solution (penicillin, 10,000 units/ml; streptomycin, 10  $\mu\text{g}/\text{ml}$ ; and amphotericin B, 25  $\mu\text{g}/\text{ml}$ ) (K.C. Biologicals, Lenexa, KS), and 0.1 mM nonessential amino acids (Gibco)

and maintained at 37°C in humidified atmosphere of 5% CO<sub>2</sub>–95% air. The EC grew to contact-inhibited monolayers with the typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin/EDTA and resuspended in fresh culture medium and passaged to 100 mm dishes for EC fractionation or into 75-cm<sup>2</sup> flasks for RNA preparation.

#### **Human Umbilical Vein Endothelial Cells (HUVEC)**

HUVEC were isolated from umbilical cords as previously described [Garcia et al., 1988; Patterson et al., 1994] by treatment with collagenase (0.1%) to detach the endothelium. The cells were plated onto gelatin-coated 25 cm<sup>2</sup> flasks and cultured with M199-medium containing 20% colostrum-free bovine serum (Sigma), heparin (2 U/ml), endothelial cell growth factor (30 µg/ml; UBI, New York), and antibiotics. Confluent cells were propagated twice by detachment with 1 ml trypsin/EDTA (0.05%/0.02%) for 1–2 min at 25°C and replating. The purity of the cells was confirmed by the typical cobblestone morphology judged routinely by light microscopy and by the presence of von Willebrand Factor as assessed by immunofluorescence microscopy.

#### **Human Dermal Microvascular Endothelial Cells (HDMVC)**

HDMVC (CC-2505, Clonetics Corp., San Diego, CA) were obtained frozen at passage 3–4 and cultured 2–3 additional passages with microvascular endothelial cell growth media (CC-3125, Clonetics).

#### **Preparation of Specific Rat Tissues**

Smooth muscle samples from adult rat were sectioned into 3 mm cubes and cubes selected with minimal connective tissue were frozen at -70°C for later analysis.

#### **Microinjection and Immunofluorescence**

BPAEC were plated onto glass coverslips and grown to confluence for microinjection of either vehicle, calyculin (Ser/Thr PPase inhibitor) or I-2 (specific PPase 1 inhibitor). Pipettes were pulled to approximately 0.1–0.2 µm diameter and the tips were filled with injection solution containing 10 mM phosphate buffer pH 7.2, 75 mM KCl, 0.1% B-mercaptoethanol, 10 mg/ml lysine-fixable

FITC-labeled Dextran 10,000 as a marker of injection, and the protein or inhibitor of interest. A constant flow was established from the tip and microinjection was performed under 40× magnification on a Leitz FS Labovert Microscope. Cells are maintained in warmed M199 media with 25 mM HEPES and 4% albumin. Penetration was assessed by the phase contrast change that occurs with successful microinjection. Three to ten clusters of five to ten cells each were injected and the cells were incubated at 37°C for an additional 10 min before fixation with 5% paraformaldehyde (10 min fixation at room temperature in the dark to preserve the FITC-dextran fluorescence). Slides were rinsed and poststained with rhodamine phalloidin (1 U/ml) to visualize F-actin. Coverslips were rinsed and mounted in Slow-Fade (Molecular Probes, Eugene, OR) and observed with a 20 or 60× objective on a Nikon/BioRad MRC024 confocal microscope. Images were processed using Meta Morph Imaging software (Universal, West Chester, PA) and printed on a thermal dye diffusion printer (Kodak, Rochester, NY).

#### **Oligonucleotide Primers**

Sense and antisense primer pairs specific for CS1 isoforms synthesized at Only DNA (Midland, TX) were designed based on published human CS1 sequences [Song et al., 1993; Barker et al., 1994; Norman and Mott, 1994] as follows: CS1α: 5' TGG TGG CCA AGT TCC TC 3' (sense) and 5' CCG TAC TTC CCC TTG TT 3' (antisense). CS1δ: 5' TGT AGT CAG TAA ATT TCT GA 3' (sense) and 5' GGT ATT TAG CTT TCT TTT C 3' (antisense). CS1γ: 5' GTG GTT GCA AAA TTT CTC C 3' (sense), and 5' CTC GTG GCA TTT GGC TTC 3' (antisense).

#### **RNA Preparation**

Total RNA from HUVEC, HDMVC, BPAEC, and rat smooth muscle was extracted by the guanidium thiocyanate method [Chomczynski and Sacchi, 1987] as we have previously described [Verin et al., 1998A]. Human uterus total RNA was purchased from Clontech Laboratories, Inc.

#### **Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Oligo(dT)-primed synthesis of first strand cDNA from HUVEC total RNA was performed using the Super Script Preamplification Sys-

tem kit from Life Technologies (Gaithersburg, MD). The same kit was employed for PCR amplification of DNA fragments ( $\approx 300$  bp each) close to the 3' end of the coding region of CS1- $\alpha$ ,  $\delta$ , and  $\Gamma$ , using isoform-specific primer pairs. Each PCR reaction (30 cycles) consisted of denaturing for 1 min at 94°C, followed by annealing for 2 min at 50°C, then extension for 2 min at 72°C. Each three PCR product was confirmed by sequencing.

#### Northern Blot Analysis

For Northern blot analysis, 20  $\mu\text{g}$  each of total RNA samples from HUVEC, BPAEC, human uterus and rat tissues were separated on 1% agarose/formaldehyde gel, blotted onto nitrocellulose membrane, and hybridized with the  $^{32}\text{P}$ -labeled CS1 isoform-specific PCR fragments.

#### BPAEC Fractionation

Myosin-enriched and myosin-depleted BPAEC fractions were prepared as we have previously described [Verin et al., 1995]. Briefly, EC monolayers were first washed twice with phosphate buffered saline (PBS, Sigma; 10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) and twice with ice-cold homogenization buffer (50 mM Tris, 0.1 mM EDTA, 28 mM  $\beta$ -mercaptoethanol, pH 7.5) containing protease inhibitors (0.5 mM PMSF, 0.1 mM TLCK, 0.1 mM leupeptin, 2 mM benzamidine). Homogenization buffer (200  $\mu\text{l}$ ) was added to the EC monolayers and plates were quickly frozen at -70°C, scraped and homogenized by passing the cell suspension several times through 1 cc tuberculin syringe, then homogenates were aliquoted and kept at -70°C. To prepare myosin-enriched EC fractions, EC homogenates were treated with 0.6 M NaCl, 0.1% Tween-20 (high-salt buffer) at 4°C for 1h followed by low-speed centrifugation (30 min, 4,500 $\times$  g at 4°C). After centrifugation, the supernatant was diluted 10 $\times$  with 50 mM Tris, 0.1 mM EDTA, 28 mM  $\beta$ -mercaptoethanol, pH 7.0 including 0.5 mM PMSF and 2 mM benzamidine and subjected to high-speed centrifugation in Eppendorf 5415 C Centrifuge at 10,000 rpm for 30 min at 4°C. The resulting pellet fraction, containing more than 95% of total extracted myosin was dissolved in high-salt buffer (myosin-enriched fraction). Less than 5% of extracted myosin was present in the supernatant (myosin-depleted fraction).

#### Immunoblotting Analysis of EC Fractions

Bovine EC fractions were next subjected to SDS-PAGE [Laemmli, 1970] on 10% gels and either stained with Coomassie blue R-250 or electrophoretically transferred to a nitrocellulose membrane as described [Towbin et al., 1979]. After transferring 18 h at 30V, the nitrocellulose membrane was blocked for 1 h in 5% nonfat dry milk in PBS, pH 7.4 including 0.1% Tween-20 (PBST), and then incubated with 1:1000 diluted antibody of interest for 1 h. Signals were detected by enhanced chemiluminescence (ECL) procedure according to the manufacturer's instructions (Amersham, Little Chalfont, Buckinghamshire, England).

#### MLCK/Myosin Immunoprecipitation

For immunoprecipitation under nondenaturing conditions confluent EC monolayers in 60-mm tissue culture dishes were rinsed once with PBS, then lysed for 30 min at 4°C with constant agitation with 500  $\mu\text{l}$  of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.5% NP-40) including protease inhibitor cocktail (0.2 mM AEBSF, 10  $\mu\text{M}$  leupeptin, 1  $\mu\text{M}$  pepstatin, 1  $\mu\text{g}/\text{ml}$  aprotinin, 1 mM benzamidine). The lysate was scraped, homogenized by passing several times through a 26-gauge needle and diluted with 500  $\mu\text{l}$  of immunoprecipitation buffer. To avoid nonspecific binding to Protein A, the cell lysates were incubated with 100  $\mu\text{l}$  of 10% Pansorbin suspension (formalin-hardened and heat-killed Cowan 1 strain *Staphylococcus aureus* cells, Calbiochem, La Jolla, CA) in immunoprecipitation buffer at 4°C for 30 min with gentle rotation. The insoluble materials were removed by microcentrifugation at 4°C for 15 min. To immunoprecipitate MLCK/ myosin complex the supernatant was incubated with 5  $\mu\text{l}$  of D119 MLCK antibody at 4°C for 1h, and then with 100  $\mu\text{l}$  of 10% Pansorbin suspension at 4°C for 1 h with gentle rotation. After microcentrifugation the pellet was washed 3 $\times$  with immunoprecipitation buffer, resuspended in 200  $\mu\text{l}$  of 2 $\times$  SDS sample buffer, heated to boiling for 5 min and microcentrifuged for 5 min. The supernatant was further used for Western immunoblotting analysis.

### Construction and Expression of pEGFP-C1-CS1 $\delta$ Plasmid

The entire coding region of CS1 $\delta$  (clone #4) was inserted into pEGFP-C1 vector (Clontech) at SalI and BamHI sites. BPAEC at 100 mm plates grown to 80% confluency were transfected with either vector or CS1 $\delta$  construct using FuGENE 6 reagent (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's protocol and GFP or GFP-CS1 $\delta$  fusion protein were immunoprecipitated under non-denaturing conditions using anti-GFP antibodies as described above.

### Protein Concentration

This was determined by the Bradford method [Bradford, 1976] using BSA as a standard.

## RESULTS

### Effect of Phosphatase I Inhibition on EC Cytoskeletal Organization

We have previously shown that treatment EC with PPase 1 inhibitors, such as calyculin, dramatically increased EC permeability and lead to gap formation which is usually dependent upon changes in EC cytoskeletal organization [Verin et al., 1995]. To further study the effect of PPase 1 on the EC cytoskeleton, we initially microinjected EC with either FITC-conjugated dextran (marker for microinjection) or mixture of calyculin and FITC-dextran. Figure 1 demonstrated that microinjection of FITC-dextran (10 mg/ml) alone have no significant effect on EC cytoskeletal organization. In contrast, cell clusters injected with calyculin (1 nM) showed an increase in cellular F-actin content and formation of gaps between adjacent cells. Consistent with these data microinjection of PPase 1-specific inhibitory protein, I-2 (1  $\mu$ M) in bovine endothelium leads to an increase in F-actin stress fibers which are organized parallel to the long axis of the cells (Fig. 2). Furthermore, in contrast to the normal apposition of the uninjected cells to one another, injected cells shifted from a regular polygonal pattern to a spindle shape pattern with gaps apparent at the cell borders. The large gap, shown on Figure 2A, is probably due to a retraction of an injected cell that subsequently lifted from the monolayer during the fixation/washing procedure. Microinjection cells with 2 mU/ml PPase 1 catalytic subunit (UBI) and FITC dextran followed by thrombin challenge

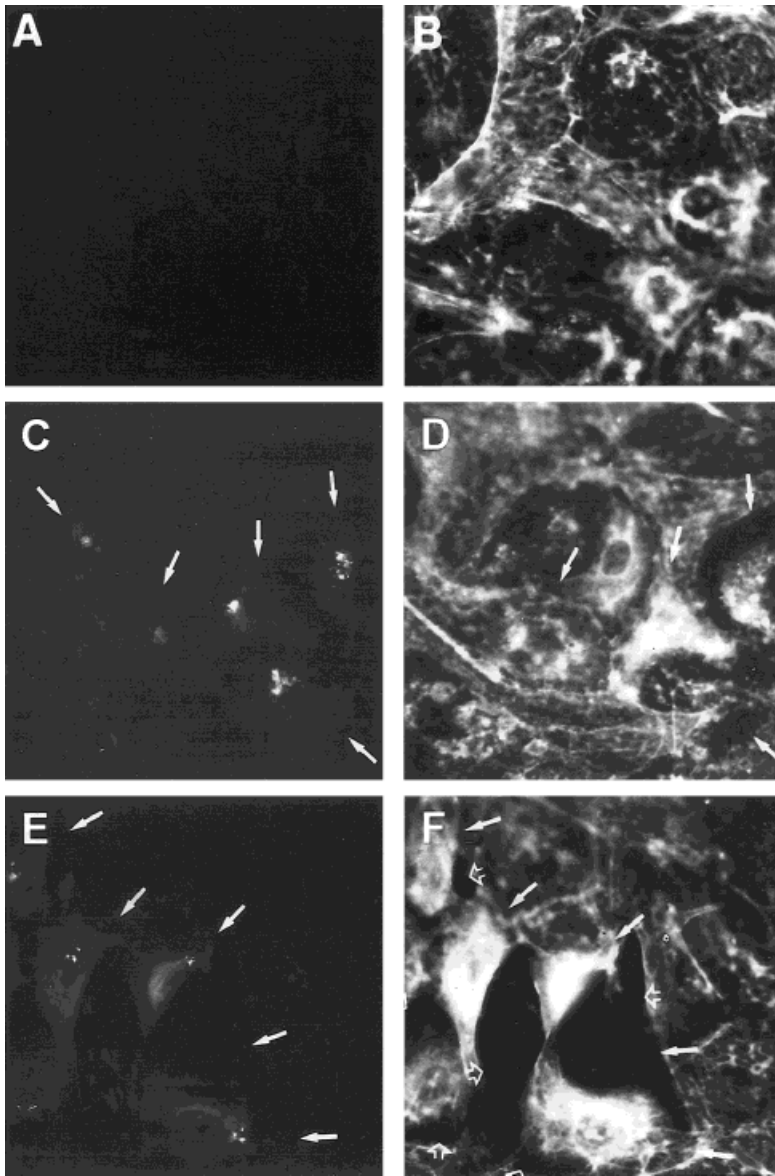
(100 nM, 10 min) shows the relative relaxation in thrombin-stimulated injected cells compared to the shape change, F-actin increase and reorganization of adjacent but uninjected cells (data not shown). These data indicate that changes in PPase 1 activity in endothelium can directly affect the EC cytoskeleton, which is consistent with data obtained in the other non-muscle cells [Fernandez et al., 1990].

### Analysis of Isoform-Specific Catalytic Subunit 1 Expression in Endothelium

The type 1 PPases characterized thus far include several catalytic (CS1) and regulatory subunits [for review, see Cohen, 1989; Shenolikar and Nairn, 1991]. Three major isoforms of CS1 ( $\alpha$ ,  $\delta$  and  $\gamma$ ) exist in eukaryotic cells [Sasaki et al., 1990], however, information is limited regarding the expression of CS1 isoforms in different cell types. To further characterize PPase 1 activity in endothelium, we next studied the expression of CS1 isoforms by reverse transcriptase polymerase chain reaction (RT-PCR) using isoform-specific oligonucleotide primers (see Materials and Methods) and HUVEC total RNA as a template. RT-PCR generated three cDNA fragments ( $\approx$ 230 bp each) with sequence of these fragments demonstrating that these fragments represent the  $\alpha$ ,  $\delta$  and  $\gamma$  EC CS1 isoforms. Using these isoform-specific cDNA fragments for Northern blot analysis we obtained a 1.8 kb  $\alpha$  transcript, a 3.4 kb  $\delta$  transcript and a 4.1 kb  $\gamma$  transcript from HUVEC and BPAEC (Fig. 3). Significant differences in the intensity of the signals between human and bovine EC may be explained by species-related difference in the sequence of cDNA fragments. Comparison of isoform expression in human smooth muscle and endothelium showed that smooth muscle cells preferentially expressed the CS1 $\delta$  isoform, whereas endothelial cells equally expressed both the CS1 $\alpha$  and  $\delta$  isoforms with less expression of CS1 $\gamma$ . The different pattern of PPase 1 expression in smooth muscle and endothelium observed may be important in the regulation of phosphorylation cascades in these tissues.

### Fractional Distribution of CS1 Isoforms in Endothelium

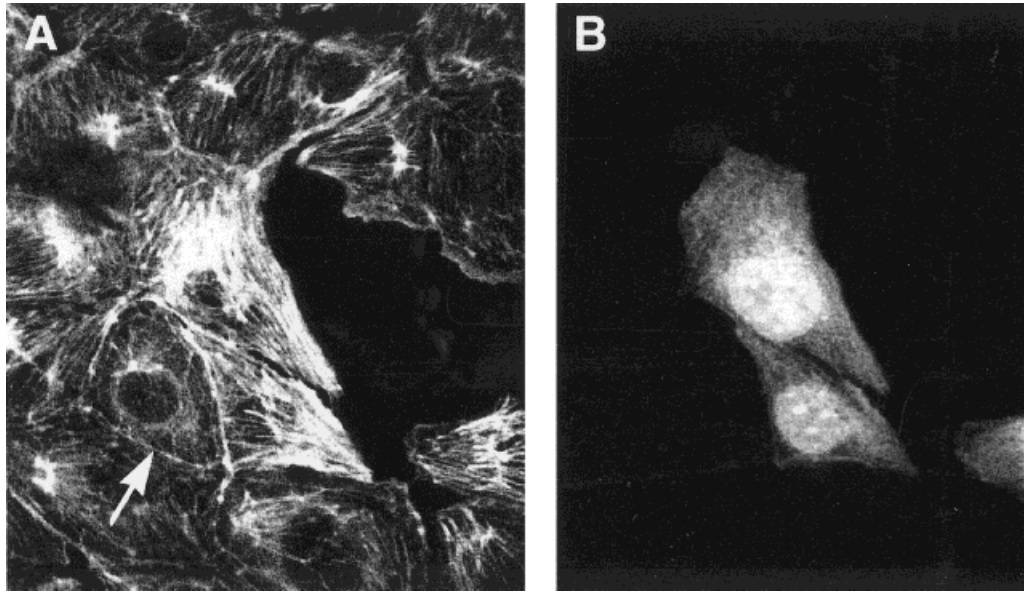
To more clearly define which CS1 isoform is associated with endothelial myosin, we screened myosin-enriched BPAEC fraction,



**Fig. 1.** Immunofluorescent images of bovine EC microinjected with calyculin. Bovine pulmonary artery EC were microinjected, fixed, and stained with rhodamine phalloidin as described in Materials and Methods. **Panels A and B:** control cells without microinjection. **Panels C and D:** cells injected with isotonic microinjection buffer containing 10 mM phosphate, pH 7.2, 75 mM KCl and 0.1% B-mercaptoethanol and 2 mg/ml lysine-fixable FITC-conjugated dextran (Molecular Probes, Eugene, OR) as a marker for injection. **Panels E and F:** cells microinjected with mixture of FITC-dextran and 1 nM calyculin in the microinjection buffer. **Panels A, C, E:** the fields of fixed cells that were excited at 488 nm with an emission filter at 522 nm to specifically image the FITC-dextran within the injected cells. **Panels B, D, F:** same images of cells after excitation at 568 nm with emission filter at 598 nm to visualize F-actin. Solid arrows show microinjected cells. Open head arrows show intercellular gaps. Magnification 20X. Clusters of cells microinjected with calyculin, but not with FITC-dextran alone showed an increase in F-actin content and intercellular gap formation.

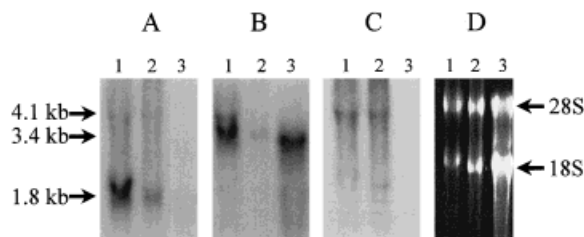
prepared as we have previously described [Verin et al., 1995] with anti-CS1 $\delta$  antibodies (UBI) and CS1 $\delta$  antibodies (gift from Dr. A. DePaoli-Roach). Comparison of the immunoreactivities of the different anti-CS1 antibodies with purified CS1 $\alpha$  and  $\delta$  isoforms shows that these antibodies do not exhibit 100% isoform specificity. However, anti-CS1 $\alpha$  antibody preferentially recognize the CS1 $\alpha$  isoform whereas the anti-CS1 $\delta$  antibody equally cross-reacts with both isoforms (Fig. 4A). The anti-CS1 $\delta$  antibody clearly cross-reacts with CS1 in myosin-enriched fraction whereas anti-CS1 $\alpha$  antibodies did not recognize any protein in this

fraction (Fig. 4A). The results of these experiments suggest that CS1 $\delta$ , but not CS1 $\alpha$  is specifically associated with endothelial myosin. Consistent with these data, CS1 $\delta$  coimmunoprecipitated with MLCK/myosin under non-denaturing conditions (Fig. 4B). Moreover, overexpression of GFP-CS1 $\delta$  construct in bovine endothelium followed by immunoprecipitation with GFP-specific antibody revealed the presence of actin and myosin in the CS1 $\delta$  non-denaturing immunoprecipitates (Fig. 4C). These data strongly suggest a stable association between CS1 $\delta$  and contractile proteins in a functional complex.



**Fig. 2.** Immunofluorescent images of bovine EC microinjected with PPase 1 inhibitor-2. BPAEC were microinjected with specific PPase 1 inhibitor (inhibitor-2, 1  $\mu$ M) and FITC-dextran (10 mg/ml). **Panel A** shows subsequent rhodamine-phalloidin staining of F-actin, indicating injected cells after 10 min have increased stress fiber formation, retraction to spindle shape, and gap formation when compared to neighboring cells. A

large gap is noted where an injected cell has retracted and then lifted from the monolayer during fixation and staining. **Panel B** shows the matched location of the injected cells by fluorescein. Cells only injected with FITC-dextran were indistinguishable from neighboring cells by rhodamine fluorescence. Magnification 60 $\times$ .

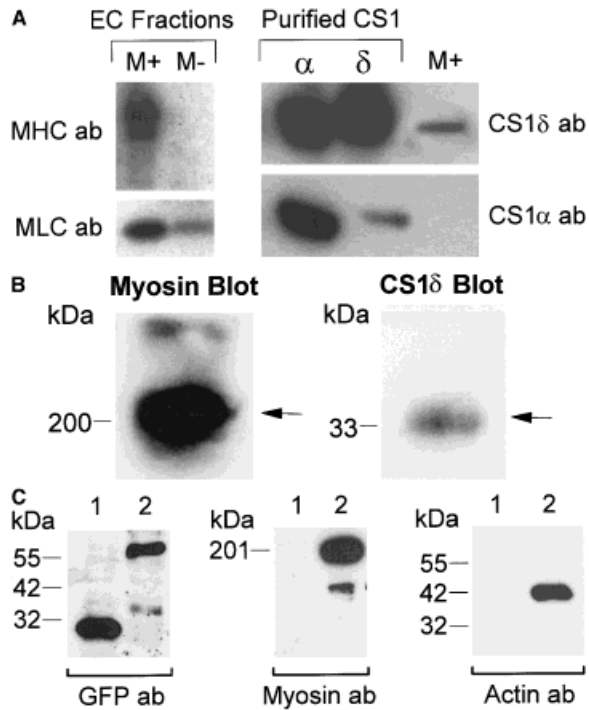


**Fig. 3.** Northern blot analysis of CS1 isoform content in human and bovine endothelium. 20  $\mu$ g each of total RNA samples from HUVEC (1), BPAEC (2), and human uterus (3) were separated on 1% agarose/formaldehyde gel and blotted onto nitrocellulose membrane. The filter was hybridized at 55 $^{\circ}$ C overnight with  $^{32}$ P-labeled DNA probes specific for CS1 $\alpha$  (A), CS1 $\delta$  (B), and CS1 $\gamma$  (C) obtained as described in Results, then washed with 0.2  $\times$  SSC at 65 $^{\circ}$ C. Positions and apparent molecular weights of RNA transcripts are indicated on the left side. **D** shows the ethidium bromide-stained gel before the transfer. The position of ribosomal RNAs are shown on the right side.

### Cloning CS1 $\delta$ in Human Endothelium

To further characterize the myosin-specific isoform of PPase 1 in endothelium, we initiated cloning of CS1 $\delta$  using a human umbilical vein endothelial cell oligo (dt)  $\lambda$  gt11 cDNA library. To generate a cDNA probe, two oligonucleotides were synthesized based on the sequence

of the rat carcinoma CS1 $\delta$  isoform [Sasaki et al., 1990]: sense, 21mer, 5'-CTC GTG AAA TCT TTC TTA GCC-3' and antisense, 21mer, 5'-CTC CGA ATC TGT TCC ATA GAC-3'. These primers were next used for RT-PCR using rat skeletal muscle total RNA as a template. The resulting 437 bp cDNA PCR product (representing bp 121–558 of the CS1 $\delta$  coding region) was used for the first library screening, yielding approximately 100 positive plaques, 10 of which were plated. Secondary screening gave 13 positive clones from which  $\lambda$  phage DNA was prepared. PCR of  $\lambda$  DNA from each clone with  $\lambda$  gt11 vector primers produced a single DNA gel band that ranged from 1.6 kb to 3.0 kb (Fig. 5A). DNA bands were purified, partially sequenced (GenBank accession no. AF092905) and compared to GenBank sequences. All sequenced fragments demonstrated high homology ( $\approx$ 99%), but not complete identity with human PPase 1 $\delta$  catalytic subunit from the NTERA 2 teratocarcinoma cell line (CS1 $\beta$  according classification of Cohen and coworkers) [Barker et al., 1994] (Fig. 5B,C). The conservation of amino acid sequence was 100% between the human endothelial cell



**Fig. 4.** Immunoblotting analysis of CS1 isoforms content in BPAEC. **Panel A:** Proteins from myosin-enriched (M+) and myosin-depleted (M-) fractions were obtained as we have previously described [Verin et al., 1995], separated by 10% SDS-PAGE followed by Western immunoblotting with either anti-MLC, myosin heavy chains (MHC), CS1 $\alpha$ , or CS1 $\delta$  antibodies. Immunoreactive protein bands were visualized by ECL technique. 100 ng of purified CS1 isoforms were used as positive controls. The amount of proteins loaded on the gel in each EC fraction was equivalent and represented  $\approx 25 \mu\text{l}$  of the initial homogenate. **Panel B:** Anti-MLCK/ myosin immunoprecipitates, which include a comparable amounts of MLCK and myosin [Verin et al., 1998A] were prepared from BPAEC extracts under nonreducing conditions with D119 antibodies and subjected to gradient (4–15%) SDS-PAGE. This was followed by Western immunoblotting with antimyosin antibodies (left panel) or CS1 $\delta$  antibodies (right panel). Each line contains 10  $\mu\text{g}$  of total protein. Positions of myosin heavy chains, CS1 $\delta$  and molecular weight markers are indicated. **Panel C:** Anti-GFP nonreducing immunoprecipitates were prepared from BPAEC transfected with either pEGFP-C1 vector (Clontech, 1) or pEGFP-C1-CS1 $\delta$  construct (2) and subjected to gradient (4–15%) SDS-PAGE. This was followed by Western immunoblotting with anti-GFP antibody (**left panel**), antimyosin antibody (**central panel**) or anti-actin antibody (**right panel**). Each line contains 10  $\mu\text{g}$  of total protein. Positions of molecular weight markers are indicated.

and human carcinoma cell coding regions. The DNA sequence of this region was also identical with only one conserved base change. In contrast, several base substitutions in the 5' non-coding region of EC CS1 $\delta$  were noted when compared to published sequence [Barker et al.,

1994] (Fig. 5C). Although the sequence of human endothelial CS1 $\delta$  in the noncoding regions is not complete, our data support several published observations indicating the highly conserved nature of CS1 isoforms in different tissues [Sasaki et al., 1990; Barker et al., 1994].

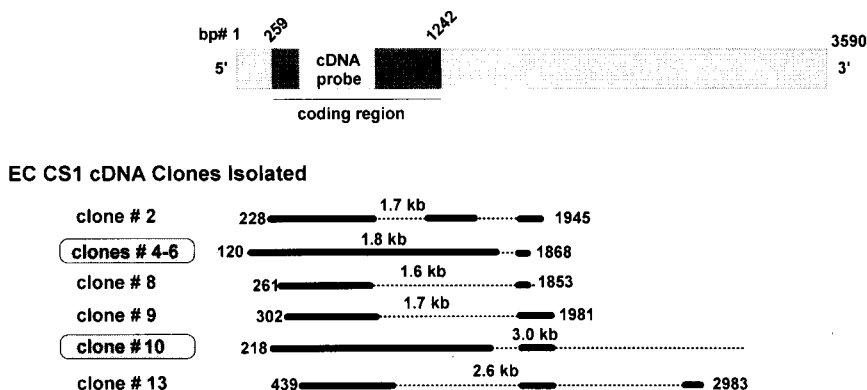
#### Expression of CS1 $\delta$ Isoform in Human Macro- and Microvascular Endothelium

We next compared the level of CS1 $\delta$  isoform expression in human macro- and microvascular endothelial cells (HUVEC and HDMVC, respectively) using Northern blotting analysis utilizing a 1.7 kb cDNA fragment (clone #4, Fig. 5A). As a positive control we hybridized the same probe with total RNA from rat smooth muscle, enriched in CS1 $\delta$ . Figure 6 indicates this analysis yielded a 3.4 kb CS1 $\delta$  transcript present in smooth muscle as well as both types of human endothelial cells. The level of CS1 $\delta$  expression is higher in human macrovascular EC, than in microvascular cells, possibly reflecting the differences in contractile responses in the two types of endothelium.

#### DISCUSSION

Using primarily an approach that utilizes pharmacologic inhibitors, we previously demonstrated that PPase 1 is directly involved in endothelial cell contractility and barrier function [Verin et al., 1995]. For example, inhibition of PPase 1 by the potent cell permeable inhibitor calyculin leads to a dramatic increase in MLC phosphorylation, which correlates with cell retraction and increased permeability of EC monolayers. It is generally accepted that changes in EC barrier are determined by changes in cytoskeletal architecture of endothelium [for review, see Lum and Malik, 1994; Garcia and Schaphorst, 1995]. We have previously shown that activation of endothelium by the coagulation protease, thrombin, leads to an increase in F-actin content, stress fiber formation and EC gap formation [Garcia et al., 1995]. Our recent published data [Verin et al., 1998B,C] demonstrated that the thrombin contractile effect can be explained, at least partially, by a combination of EC MLCK activation and PPase 1 inhibition. Both events serve to increase MLC phosphorylation, actin redistribution and initiation of contraction. The present study extends these findings and demonstrates that, similar to thrombin, microinjec-



**A Published Structure of Human Teratocarcinoma CS1 $\delta$** 

**Fig. 5.** Cloning of CS1 $\delta$  in human endothelium. **Panel A:** Schematic representation of the CS1 cDNA and endothelial cell CS1 cDNA clones isolated. Open bar indicated position of cDNA probe used for library screening. The sequenced regions of the endothelial cell CS1 $\delta$  clones are represented by solid lines. Dashed lines show the approximate size of each

clone. Clones, which encode the entire coding region are shown in circles. **Panel B:** The combined oligonucleotide and deduced amino acid sequence of human endothelial CS1 $\delta$  clones isolated. **Panel C:** Sequenced regions of endothelial cell CS1 $\delta$  differ from the published human sequence.

tion of both PPase inhibitor calyculin and specific PPase 1 inhibitory protein I-2 [Shenolikar and Nairn, 1991] can induce stress fiber formation and cell retraction. Rapid microfilament reorganization induced by Ser/Thr PPase inhibitors has been observed in several non-muscle types including neutrophils, fibroblasts, hepatocytes, and leukemia cells [Eriksson et al., 1989; Hirano et al., 1992; Kreinbuhl et al., 1992; Kurosaki et al., 1993]. In contrast to microinjection of PPase 1 inhibitors, microinjection of PPase 1 catalytic subunit in thrombin-activated EC resulted in stress fiber dissolution. This data suggests that, similar to smooth muscle cells, modulating PPase 1 activity in endothelial cells directly alters actomyosin filament organization and is consistent with published data [Fernandes et al., 1990] in fibroblasts that PPase microinjection can disassemble the actin microfilament network.

The PPase 1 responsible for the regulation of actomyosin interaction in smooth muscle has been recently purified as a trimer containing a myosin-binding 130 kDa subunit (M130), a 20 kDa subunit with unknown function (M20) and the catalytic subunit (CS1) [Shirazi et al., 1994]. It is important to note that all three major CS1 isoforms—CS1 $\alpha$ , CS1 $\delta$ , and CS1 $\gamma$ —exhibit 90% identity, similar functional characteristics [Sasaki et al., 1990; Zhang et al., 1993], and are capable of forming a complex

with M130 in vitro [Alessi et al., 1993]. However, these findings do not preclude the possibility that the structural differences in these isoforms confer subtle but potentially functionally significant differences [Zhang et al., 1993]. For example, the C-terminal region can effect the enzymatic properties of rabbit muscle PPase 1 [Martin et al., 1991]. Isoform-specific function may also serve to provide tissue-, developmental-, or cell cycle-specific expression via genetic regulation [Zhang et al., 1993]. The CS1 $\gamma$ 2 isoform, a variant of CS1 $\gamma$  isoform produced by alternative splicing [Sasaki et al., 1990], is highly expressed in testes [Kitagawa et al., 1990], whereas expression of the CS1 $\alpha$  may be elevated in hepatocarcinogenesis [Sasaki et al., 1990; Kitamura et al., 1991]. Our data indicate that human endothelial cells expressed equal amounts of CS1 $\alpha$  and CS1 $\delta$  isoforms with much less expression of CS1 $\gamma$ . It is difficult to estimate the level of expression of CS1 isoforms in bovine endothelium, because human cDNA probes appeared to be less effective for bovine RNA and bovine CS1 sequences are not available. We conclude that, similar to HUVEC, all three CS1 isoforms are present in bovine EC, however, in contrast, our data indicate that human smooth muscle cells (human uterus) express only CS1 $\delta$ . Information is limited about expression of CS1 isoforms in different groups of muscle tissues. Nonquantitative immunocytochemical analysis revealed the

**B**

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1 CCTGCCTCAG CCTCCGAGC AGCCGGGGTC GAAACGCCGC GTGACTTGTA GGTGAGAGAA
61 CGCCGAGCCG TCGCCGCGAGC CTCCGCGGCC GAGAAGCCCT TGTTCCTGCT GCTGGGAAGG
121 AGAGTCTGTG CCGACAAGAT GCGGGACGGG GAGCTGAACG TGGACAGCCT CATCACCCGG
      M A D G E L N V D S L I T R 14

181 CTGCTGGAGG TACGAGGATG TCGTCCAGGA AAGATTGTGC AGATGACTGA AGCAGAAGTT
      L L E V R G C R P G K I V Q M T E A E V 34

241 CGAGGCTTAT GTATCAAGTC TCGGGAGATC TTTCTCAGCC AGCCTATTCT TTTGGAATTG
      R G L C I K S R E I F L S Q P I L L E L 54

301 GAAGCACCGC TGAAAATTG TGGAGATATT CATGGACAGT ATACAGATTT ACTGAGATTA
      E A P L K I C G D I H G Q Y T D L L R L 74

361 TTTGAATATG GAGGTTTCCC ACCAGAAGCC AACTATCTTT TCTTAGGAGA TTATGTGGAC
      F E Y G G F P P E A N Y L F L G D Y V D 94

421 AGAGGAAAGC AGTCTTTGGA AACCATTTGT TTGCTATTGG CTTATAAAAT CAAATATCCA
      R G K Q S L E T I C L L L A Y K I K Y P 114

481 GAGAACTTCT TTCTCTTAAG AGGAAACCAT GAGTGTGCTA GCATCAATCG CATTATGGA
      E N F F L L R G N H E C A S I N R I Y G 134

541 TTCTATGATG AATGCAAACG AAGATTTAAT ATTA AATTGT GGAAGACCTT CACTGATTGT
      F Y D E C K R R F N I K L W K T F T D C 154

601 TTTAACTGTC TGCCTATAGC AGCCATTGTG GATGAGAAGA TCTTCTGTTG TCATGGAGGA
      F N C L P I A A I V D E K I F C C H G G 174

661 TTGTCACCAG ACCTGCAATC TATGGAGCAG ATTCGGAGAA TTATGAGACC TACTGATGTC
      L S P D L Q S M E Q I R R I M R P T D V 194

721 CCTGATACAG GTTTGCTCTG TGATTGCTA TGGTCTGATC CAGATAAGGA TGTGCAAGGC
      P D T G L L C D L L W S D P D K D V Q G 214

781 TGGGGAGAAA ATGATCGTGG TGTTCCTTT ACTTTTGGAG CTGATGTAGT CAGTAAATTT
      W G E N D R G V S F T F G A D V V S K F 234

841 CTGAATCGTC ATGATTTAGA TTTGATTTGT CGAGCTCATC AGGTGGTGG AAGATGGATAT
      L N R H D L D L I C R A H Q V V E D G Y 254

901 GAATTTTTTTG CTAAACGACA GTTGGTAACC TTATTTTTCAG CCCCAAATTA CTGTGGCGAG
      E F F A K R Q L V T L F S A P N Y C G E 274

961 TTTGATAATG CTGGTGGAAAT GATGAGTGTG GATGAACTT TGATGTGTTT ATTTTCAGATA
      F D N A G G M M S V D E T L M C S F Q I 294

1021 TTGAAACCAT CTGAAAAGAA AGCTAAATAC CAGTATGGTG GACTGAATTC TGGACGTCCT
      L K P S E K K A K Y Q Y G G L N S G R P 314

1081 GTCACTCCAC CTCGAACAGC TAATCCGCCG AAGAAAAGGT GAAGAAAGGA ATTCTGTAAA
      V T P P R T A N P P K K R STOP 327

1141 GAAACCATCA GATTTGTTAA GGACATACTT CATAATATAT AAGTGTGCAC TGTA AAACCA
1201 TCCAGCCATT TGACACCCTT TATGATGTCA CACCTTTAAC TTAAGGAGAC GGGTAAAGGA
1261 TCTTAAATTT TTTTCTAATA GAAAGATGTG CTACACTGTA TTGTAATAAG TATACTCTGT
1321 TATAGTCAAC AAAGTTAAAT CCAAATTC AAATTATCCAT TAAAGTTACA TCTTCATGTA
1381 TCACAATTTT TAAAGTTGAA AAGCATCCCCA GTTAAACTAG ATGTGATAGT TAAACCATGAT
1441 GAAAGCATGA TGATCCATCT GTGTAATGTG GTTTTAGTGT TGCTTGGTTG TTTAATTATT
1501 TTGAGCTTGT TTTGTTTTTG TTTGTTTTCA CTAGAATAAT GGCAAATACT TCTAATTTT
1561 TTCCTAAAC ATTTTAAAAA GTGAAATATG GGAAGAGCTT TACAGACATT CACCAACTAT
1621 TATTTTCCCT TGTTTTATCTA CTTAGATATC TGTTTTAATCT TACTAAGAAA ACTTTTCGCT
1681 CATTACATTA AAAAGGAATT TTAGAGATTG ATTTGTTTTAA AAAAAATAC GCACATTGTC
1741 CAATCCA

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**C**

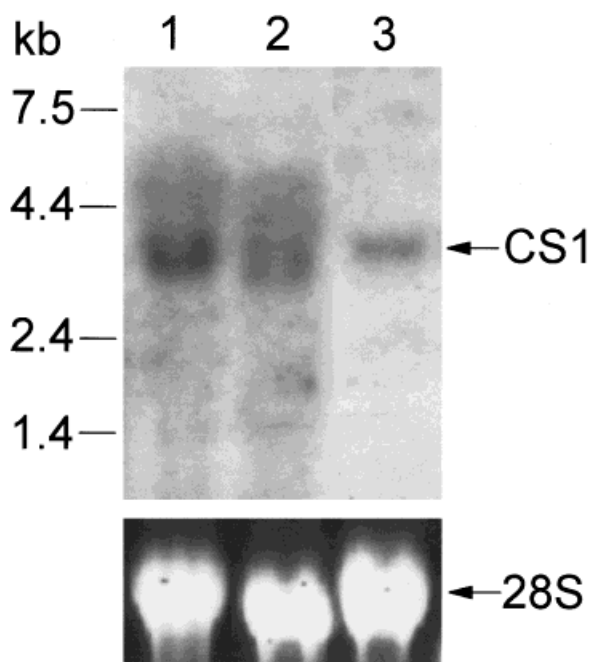
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HSPPP1CB 121 CCGGGAAAAGGGGAGTTGGAGCCGGGG 148
          ||| ||| ||| ||| ||| ||| |||
CS1Delta 1 CCTGCCTCAGCCTCCCAGCAGCCGGGG 28

HSPPP1CB 452 ATGGACAATATACAG 466
          ||| ||| ||| ||| ||| |||
CS1Delta 332 ATGGACAGTATACAG 346

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Figure 5. (Continued.)



**Fig. 6.** Northern blot (**upper panel**) of human umbilical vein (macrovascular, 1), human dermal (microvascular, 2) endothelial and rat smooth muscle (3) RNA probed with clone #4 cDNA, which contains the complete coding region of human EC CS1 $\delta$  (see Fig. 5). Samples of 20  $\mu$ g of total RNA were blotted and hybridized with the 1.7 kb cDNA fragment (clone #4) overnight at 65°C and then washed with 0.2 $\times$  SSC buffer. **Lower panel** shows the images of 28S ribosomal RNA from ethidium bromide-stained gel of the same samples. Positions of molecular weight markers, ribosomal RNA, and CS1 transcript are indicated.

presence of both CS1 $\alpha$  and CS1 $\delta$  isoforms in rat aortic smooth muscle cells [Murata et al., 1997], whereas CS1 $\delta$  isoform was about 4.6-fold more abundant than CS1 $\alpha$  in human skeletal muscle as it was evidenced by quantitation of Northern blotting [Barker et al., 1994]. CS1 $\delta$ , but not CS1 $\alpha$ , was recently shown in smooth muscle to be associated with M130 and M20 in a trimeric functional complex during myosin-associated PPase 1 purification [Shirazi et al., 1994]. However, in permeabilized cells the CS1 $\alpha$  isoform was colocalized with M130 in smooth muscle cells and fibroblasts, whereas CS1 $\delta$  was found in focal adhesions [Murata et al., 1997]. Information is limited regarding the structure of myosin-associated PPase 1 in endothelium. We have recently shown that in contrast to smooth muscle, myosin-associated PPase 1 endothelium may exhibit two distinct myosin-specific PPase 1 regulatory subunits (M70 and M110) that

share certain structural features with the M130 regulatory subunit from smooth muscle and tightly associated with contractile proteins in a functional complex [Verin et al., 2000]. Our present results indicate that only the CS1 $\delta$  isoform is found in the myosin-enriched EC fraction as well as in non-denaturing EC MLCK/myosin immunoprecipitates, which include comparable amounts of MLCK and myosin [Verin et al., 1998A]. Consistent with these data overexpressed GFP-CS1 $\delta$  was tightly associated with actomyosin. Together, these data provide strong evidence that CS1 $\delta$ , but not CS1 $\alpha$ , is associated with myosin-based contractile protein complex in endothelium.

To further examine myosin-associated PPase 1 in endothelium, we partially cloned CS1 $\delta$  from a HUVEC library we have previously utilized successfully to clone EC MLCK [Garcia et al., 1997]. Sequencing analysis demonstrated that human endothelial CS1 $\delta$  is  $\approx$ 99% homologous with the previously published sequence of human teratocarcinoma CS1 $\delta$  (CS1 $\beta$  according to classification of Cohen and coworkers [Barker et al., 1994]. These results are consistent with high-sequence conservation of the PPase 1 catalytic subunit between species and cell types [Sasaki et al., 1990; Barker et al., 1994]. We also studied the expression of CS1 $\delta$  in different types of endothelium (macro vs. micro) using a 1.8 kb cDNA fragment of cloned EC CS1 $\delta$  as a probe for Northern blotting. Our results indicate that level of expression of CS1 $\delta$  in human macrovascular endothelium is  $\approx$ two-fold higher than in human microvascular EC. Although information is limited regarding differences in the contractile regulation between macro- and microvascular endothelium, morphological and biochemical differences between macro- and microvascular EC have been observed [Kelly et al., 1998]. For example, microvascular EC are smaller and form more homogeneous monolayers, more intact tight junctions, and produce  $\approx$ tenfold higher transmonolayer endothelial resistance compare to macrovascular cells [Blum et al., 1997]. Recently, several distinct patterns of EC actin filaments were observed depending on the caliber and type of vessel using confocal microscopy [Thurnston and Baldwin, 1994]. Our recently published data indicate that both micro- and macrovascular EC express the novel 214 kDa MLCK isoform, which is distinct from smooth muscle MLCK [Verin et al., 1998B]. It was recently

found [Diwan et al., 1997] that in rat pulmonary microvascular EC, phosphatase inhibitors, like calyculin, did not cause actin-myosin colocalization as was reported for human macrovascular cells (HUVEC) after thrombin treatment [Goekeler and Wysolmerski, 1995]. While it is interesting to speculate that difference in the contractile response in macro- and microvascular endothelium may depend, at least in part, on differential expression of the myosin-associated CS1 $\delta$ , further studies are required to more clearly define distinct biochemical regulatory mechanisms that exist in macro- and microvascular endothelium.

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