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Immunochemical Characterization of Myosin-Specific Phosphatase 1 Regulatory Subunits in Bovine Endothelium

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Abstract We have previously shown that myosin-specific phosphatase 1 (PPase 1) activity is critical for maintaining endothelial cell barrier function (Verin et al. [1995] Am. J. Physiol. 269:L99–L108). To further characterize myosin-specific PPase 1 in endothelium, we generated antibodies specific to published sequences of the myosin-associated PPase 1 regulatory subunit (M110) from smooth muscle. Peptide antigens were designed based upon consensus sequences for a single ankyrin repeat (ANK 110) and a leucine zipper motif region (LZ 110), which represents putative sites for binding the PPase 1 catalytic subunit (CS1) and myosin, respectively. Our initial study demonstrated that each antibody immunoprecipitated 2 proteins with an apparent Mr of 110 and 70 kD on SDS-PAGE. The CS1δ isoform, which appeared to be characteristic for the myosin-specific phosphatase, was co-immunoprecipitated under non-denaturing conditions with ANK110 and LZ110 as was actin, myosin, and myosin light chain kinase (MLCK). Similarly, immunoprecipitation with specific anti-myosin or anti-MLCK antibodies under the same conditions, followed by immunostaining with either LZ110 or ANK110 revealed the same 110 and 70 kD protein bands. The 70 kD protein (p70) was immunoreactive with ANK 110 and LZ 110, was complexed with myosin and MLCK, and was detected in non-denaturing M110 immunoprecipitates. Consistent with these results, endothelial cell fractionation demonstrates the presence of p70 in both cytoskeletal and myosin-enriched fractions, but not in the myosin-depleted (cytosolic) fractions. These data suggest that endothelial cells may exhibit two distinct myosin-specific PPase 1 regulatory subunits which share certain structural features with the M110 regulatory subunit from smooth muscle and which are tightly associated with myosin and MLCK in a functional complex. J. Cell. Biochem. 76:489–498, 2000.

Key words: phosphatase 1 regulatory subunits; myosin; endothelium

A major function of the vascular endothelial cell (EC) monolayer is to serve as a semi-selective barrier to fluid and solute flux across the blood vessel wall. Increased endothelial permeability is a prominent feature of inflammatory syndromes and is the result of intercellular gap formation [Garcia et al., 1986, 1995] under close regulation by contractile forces [Garcia and Schaphorst, 1995; Garcia et al., 1996]. Contractile activity appears to be controlled, at least in part, by the level of myosin phosphorylation, which is determined by the activity balance between myosin light chain kinase (MLCK) and myosin-specific phosphatase (PPase) activities [for review see De Lannerole and Paul, 1991; Somlyo and Somlyo, 1994]. Although we have recently characterized a novel, large molecular weight isoform of MLCK (214 kD) in cultured endothelium [Garcia et al., 1997; Verin et al., 1998a,b], understanding remains limited as to the PPases involved in regulation of MLC phosphorylation. Ser/Thr protein PPases (type 1 PPase, and three type 2 enzymes, PPase 2A, PPase 2B, and PPase 2C) can be distinguished on the basis of substrate specificity, divalent cation requirements and susceptibility to selective PPase inhibitors [for review see Cohen, 1989; Shenolikar and Nairn, 1991]. Primary structure analysis shows that all catalytic subunits, except the PPase 2C, belong to a single gene family with a high degree of conservation among isoforms [for review see Shenolikar and

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Nairn, 1991; Mumby and Walter, 1993]. Nevertheless, at least two isoforms of each Ser/Thr PPase are present in mammalian cells [Shenolikar and Nairn, 1991; Cohen, 1990]. All four types of Ser/Thr PPases are able to effectively dephosphorylate MLC in vitro [Ingebritsen and Cohen, 1983; Stewart et al., 1983; Pato et al., 1983]. Recently, myosin-associated PPases 1 from skeletal and smooth muscle were purified and found to contain CS1δ as a major isoform [Alessi et al., 1992]. More importantly, however, distinct regulatory subunits, which target the myosin-specific PPase toward myosin [Alessi et al., 1992; Shirazi et al., 1994; Dent et al., 1992], were identified in skeletal muscle (60 kD) and smooth muscle (20 kD and 130 kD) [Alessi et al., 1992; Shirazi et al., 1994; Dent et al., 1992]. Association of the smooth muscle myosin-binding complex, but not skeletal muscle regulatory subunit, to CS1 greatly enhanced PPase activity toward smooth muscle myosin [Alessi et al., 1992; Shirazi et al., 1994; Dent et al., 1992].

These results suggest that the substrate specificity of PPase from smooth muscle and skeletal muscle tissues differs significantly and highlight the critical need to explore the role of endothelial cell-specific myosin PPase activities in EC contractility. Using pharmacological inhibitors we demonstrated direct involvement of PPase 1 in the regulation of EC gap formation and barrier function in pulmonary artery endothelium [Verin et al., 1995]. Consistent with these data, PPase 1, but not 2A, appeared to play a major role in the regulation of pulmonary microvascular EC barrier function [Diwan et al., 1997]. Immunocytochemical data and inhibitory analysis indicated that PPase 1 is involved in sustaining the normal cytoskeletal structure in umbilical vein endothelium [Shinoki et al., 1995]. Recent studies also demonstrated that endothelial cell activation by thrombin leads to inhibition of myosin-associated PPase 1 activity [Essler et al., 1998; Shasby et al., 1997; Verin et al., 1998]. This process is mediated by Rho kinase-induced phosphorylation of M_{110} regulatory subunit [Essler et al., 1998] and caused dissociation of CS 1 from an actomyosin functional protein complex [Verin et al., 1998]. Because the structural features and mechanisms of regulation of endothelial cell PPase 1 remain unknown, in this study we have focused on the immunochemical characterization of myosin-specific PPase 1 regulatory subunit in bovine endothelium.

MATERIALS AND METHODS

Materials

Polyclonal anti-PPase 1 M110 antibody, anti-DARPP-32 and non-muscle anti-myosin antibody were purchased from Serotec Inc. (Raleigh, NC), Chemicon (Temecula, CA), and from Biomedical Technologies Inc. (Stoughton, MA), respectively. Antiserum against CS1δ and against MLCK (D119 antibody) were kindly provided by Drs. Anna DePaoli-Roach and Patricia Gallagher (Indianapolis, IN). Antibodies ANK 110 and LZ 110 were produced against peptide sequences GHVEVVKLLLDNGADVNAPT and LKSDNQRLKDENGALIRVISKL, respectively, by Biodesign International (Kennebunk, ME). 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 passages 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 µg/ml endothelial cell growth supplement (Collaborative Research, Bedford, MA), 1% antibiotic and antimycotic solution (penicillin, 10,000 units/ml; streptomycin, 10 µg/ml; and amphotericin B, 25 µg/ml; K.C. Biologicals, Lenexa, KA), and 0.1 mM non-essential amino acids (Gibco) and maintained at 37°C in humidified atmosphere of 5% CO_2-95% 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.

Immunoprecipitation Under Non-Denaturing Conditions

For immunoprecipitation 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 µl 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 µM leupeptin, 1 µM pepstatin, 1 µg/ml aprotinin, 1 mM benzamidine). The lysate was scraped, homogenized by passing several times through a 26 gauge needle and diluted with 500 µl of immunoprecipitation buffer. To avoid non-specific binding to Protein A, the cell lysates were incubated with 100 µl 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.

The supernatant was incubated with 5 µl of antibody of interest at 4°C for 1 h, and then with 100 µl of 10% Pansorbin suspension at 4°C for 1 h with gentle rotation. After microcentrifugation the pellet was washed 3× with immunoprecipitation buffer, resuspended in 200 µl of 2× SDS sample buffer [Laemmli, 1970], heated to boiling for 5 min, and microcentrifuged for 5 min. The supernatant was further used for Western immunoblotting analysis.

Prior to immunoprecipitation with M110 antibody (Serotec), antibody were immobilized on Affi-Prep Hz Hydrazide Support (Bio Rad) according following protocol. 60 µl of sheep anti M110 antibody (Serotec) were dialyzed against 1,000 volumes of oxidation buffer (0.02 M sodium acetate, 0.15 M NaCl, pH 5.0) at 4°C overnight and then oxidized by 1:50 volume of 0.5 M sodium periodate. The oxidation reaction was performed in the dark at room temperature for 1 h and stopped by addition of 1:20 volume of glycerol and subsequent mixing for 10 min at room temperature. The oxidized antibody was buffer exchanged by dialyzing against 1,000 volumes of coupling buffer (0.1 M sodium acetate, 1.0 M NaCl, pH 4.5) at 4°C overnight. A total of 800 µl of Affi-Prep Hz Hydrazide Support (Bio Rad) was washed two times with 10 volumes of H_2O and two times with 10 volumes of coupling buffer. The buffer above the settled support was removed and the support was mixed with oxidated antibody. Coupling reaction was performed in dark with adequate, but gentle mixing for 24 h. After that the support was washed three times with three volumes of phosphate buffer (0.02 M sodium phosphate, 0.5 M NaCl, pH 7.0) and stored in phosphate buffer containing 0.02% sodium azide at 4°C. About 4 µg of antibody was finally linked to carrier (Affi-Prep Hydrazide Support). For immunoprecipitation the supernatant of cell lysate obtained as described above was microcentrifuged for 15 min and then mixed with 250 µl immunoprecipitation buffer and 250 µl of immobilized antibody suspension diluted in 250 µl of immunoprecipitation buffer. The mixture was incubated at 4°C overnight with gentle agitation. The immunoprecipitated complex was harvested by microcentrifugation and solubilized in SDS buffer as described above.

BPAEC Fractionation

Actin-enriched, 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 two times with ice-cold homogenization buffer (50 mM Tris, 0.1 mM EGTA, 28 mM β-mercaptoethanol, pH 7.5) containing proteinase inhibitors (0.5 mM PMSF, 0.1 mM TLCK, 0.1 mM leupeptin, 2 mM benzamidine). Homogenization buffer (200 µl) 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 EC fractions, EC homogenates were treated with 0.6 M NaCl, 0.1% Tween-20 (high-salt buffer) at 4°C for 1 h followed by low-speed centrifugation (30 min, 4,500*g* at 4°C). After centrifugation, the pellet, containing mainly actin and actin-associated proteins (actin-enriched fraction), was rinsed twice with PBS and dissolved in SDS sample buffer [Laemmli, 1970]. The supernatant was diluted twice with 50 mM Tris, 0.1 mM EGTA, 28 mM β-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).

Cytoskeletal and cytosolic fractions were prepared as we have previously described [Verin et al., 1998c]. Briefly, confluent BPAEC from 100-mm dishes were rinsed twice with 2 ml of phosphate buffered saline (PBS, Sigma; 10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) at room temperature and incubated with 1.5 ml of extraction buffer (1% NP-40, 150 mM NaCl, 50 mM NaF, 28 mM β-mercaptoethanol in 50 mM Tris HCl, pH 8.0), containing proteinase inhibitors (0.5 mM PMSF, 0.1 mM TLCK, 0.1 mM leupeptin, 2 mM benzamidine) for 30 min at 4°C. Extracts were clarified by microcentrifugation and extractable proteins were precipitated by adding 100% ice-cold TCA. After microcentrifugation for 5 min the pellets (detergent-soluble cytosolic fraction) were washed three times with diethyl ether and solubilized in 3 ml of SDS sample buffer [Laemmli, 1970]. Insoluble proteins remaining on dishes (i.e., detergent-insoluble cytoskeletal EC fractions) were rinsed twice with ice-cold PBS and solubilized by scraping dishes in 3 ml of SDS sample buffer [Laemmli, 1970]. Endothelial cell fractions were subjected to Western immunoblotting analysis as described below.

Immunoblotting Analysis

Bovine EC fractions or immunoprecipitates 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 17–18 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 1:1,000 diluted antibodies against proteins of interest dissolved in PBST with 5% BSA for 1 h. Signals were detected by enhanced chemiluminescence (ECL) procedure according manufacturer's instructions (Amersham, Little Chalfont, Buckinghamshire, England).

Protein Concentration

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

RESULTS

Expression of Myosin-Associated PPase 1 Regulatory Subunit(s) in Bovine Endothelium

Cloning of a cDNA encoding regulatory subunits of smooth muscle PPase (M21 and M110 according to actual molecular size) revealed the existence of two specific regions in their sequences. One of these so-called "zipper motifs" (present in both subunits) shows similarity to several structural proteins and appears to be myosin-binding region whereas another region, located in the N-terminal part of M110 subunit, contains seven 33 amino acid ankyrin repeats and is likely to include the CS1 binding site [Chen et al., 1994]. For preliminary characterization of endothelial PPase 1 myosin-specific regulatory subunits, we have generated antibodies specific to the zipper motif and to the ankyrin repeat sequences. Peptide antigens were designed based upon consensus sequences for a single ankyrin repeat (ANK 110) and a leucine zipper motif region (LZ 110) of smooth muscle M110 regulatory subunit (Fig. 1) [Chen et al., 1994]. We next utilized a rationale that if these antibodies recognize a similar protein(s) in endothelial cell extracts or in myosin immunoprecipitates, it will be most likely represent the myosin-associated regulatory subunit(s) (RS) of endothelial cell PPase 1. Immunoprecipitation of ankyrin- and zipper motif-containing proteins from BPAEC cell extracts by LZ 110 and ANK 110 antibodies followed by Western immunoblotting analysis with the same antibodies revealed two protein bands with apparent molecular weights 110 and 70 kD, which cross-react with both antibodies (Fig. 2). The same protein bands were detected in myosin and MLCK non-denaturing immunoprecipitates, but not in the DARPP immunoprecipitates (nuclear PPase 1 regulatory protein) [Shenolikar and Nairn, 1991] (Fig. 2). Consistent with these data, the CS1δ isoform which appeared to be characteristic for myosin-specific PPase 1 [Shimizu et al., 1994; Shirazi et al., 1994] co-immunoprecipitated under non-denaturing conditions with ANK110 and LZ 110 (Fig. 2), as did actin and myosin (Fig. 3) and myosin light chain kinase (MLCK), but not DARPP (data not shown). To specifically immunoprecipitate M110 from endothelium, we used anti M110 antibody raised against myosin-associated chicken gizzard PPase 1 holoenzyme (Fig. 1; Serotec). Surprisingly, this antibody failed to immunoprecipi-

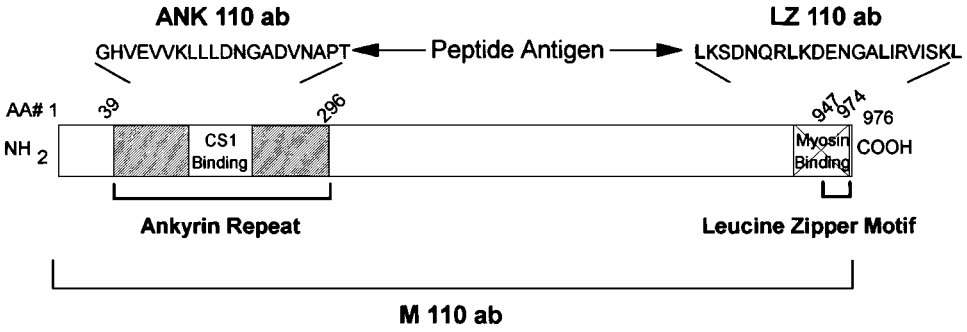

Fig. 1. Schematic structure of the rat aorta smooth muscle PPase 1 regulatory subunit (M110). Diagonal bars indicate ankyrin repeat region (hypothetical CS1 binding site) and criss-cross bar indicates hypothetical myosin-binding site, which include leucine zipper motif region based on Chen et al. [1994]. Peptide antigens were designed based upon consensus sequences for single ankyrin repeat and leucine zipper motif structures. Polyclonal antipeptide antibody (ANK 110 and LZ 110, respectively) was produced in rabbit by Biodesign. Sheep polyclonal anti M110 ab were raised against purified chicken M 110 (Serotec).

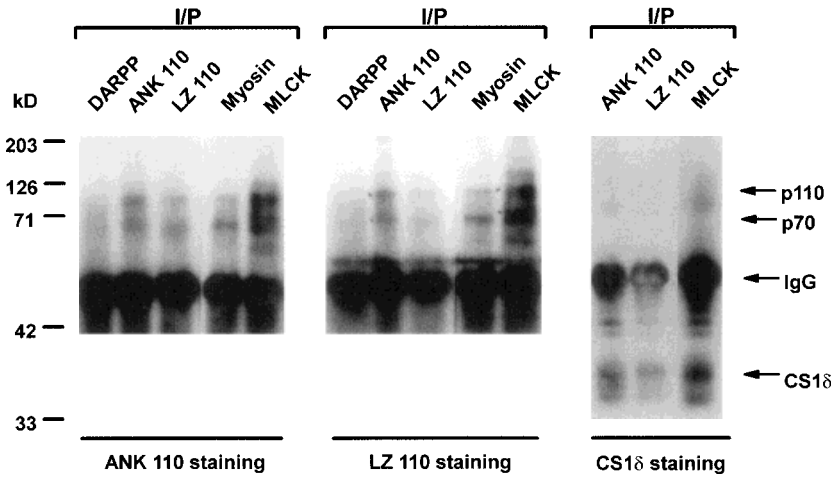

Fig. 2. Analysis of myosin-specific PPase content in endothelium. Immunoprecipitates with different antibodies (ANK 110, LZ 110, myosin, MLCK, and DARPP) prepared under non-denaturing condition as described in Materials and Methods were analyzed by immunoblotting with ANK 110 (**left**), LZ110 (**middle**), and CSIδ antibody (**right**). Immunoreactive protein bands were visualized by ECL technique. Positions of putative EC myosin PPase 1 regulatory subunits (p70 and p110), CS1δ and molecular weight markers are indicated. Immunoprecipitates with ANK 110 and LZ 110 revealed two proteins with apparent Mr 110 and 70 kD on SDS-PAGE. Both of these two proteins co-immunoprecipitate with myosin, MLCK, and CS1δ, but not with inhibitory subunit of nuclear PPase 1 (DARPP, negative control)

tate any protein within the molecular weight range characteristic for smooth muscle myosin-specific PPase 1 regulatory subunit (110–130 kD), however, they immunoprecipitated a 70 kD protein which cross-reacts with both LZ 110 and ANK 110 antibodies (Fig. 4). This protein co-immunoprecipitated with several contractile proteins including myosin and MLCK, but not with DARPP or PPase 1 inhibitor-1 known as I-1, (antibody against DARPP also recognizes I-1; Fig. 4). The results of these experiments suggest that endothelial cells have both a conventional M110 PPase 1 regulatory subunit similar to smooth muscle as well as a novel myosin-associated PPase 1 regulatory subunit (M70), which shares some structural features but is distinct from the smooth muscle PPase 1 regulatory subunit. Both endothelial cell M110 and M70 appear to be tightly associated with myosin and MLCK in a functional complex.

Fractional Distribution of M70 in Endothelium

To examine subcellular localization of M70 in endothelium we isolated actin-enriched, myosin-enriched, and myosin-depleted BPAEC fractions. We have previously shown that the actin-

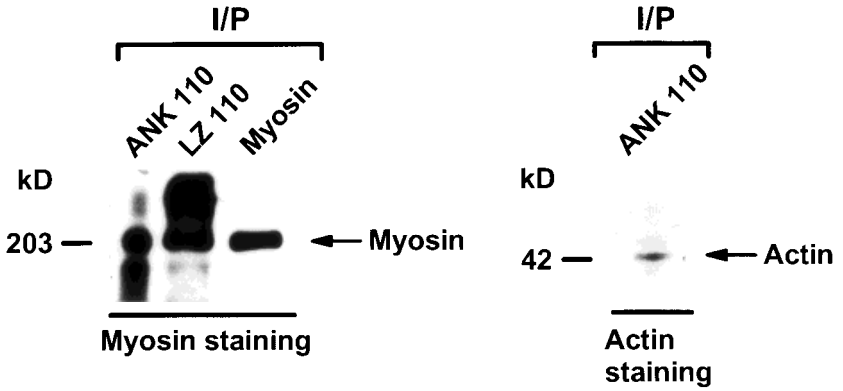

Fig. 3. Analysis of contractile protein content in ANK 110 and LZ 110 immunoprecipitates. Immunoprecipitates with different antibodies (ANK 110, LZ 110, myosin) prepared under denaturing condition as described in Materials and Methods were analyzed by immunoblotting with myosin (**left**) and actin (**right**) antibodies. Myosin, actin, and MLCK (data not shown) immunoprecipitates with ANK 110 and LZ 110 suggesting a functional complex between contractile protein and PPase 1.

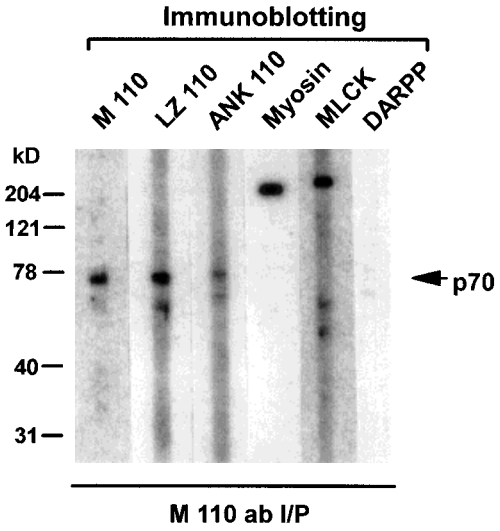

Fig. 4. Multiscreen analysis of M110 non-denaturing immunoprecipitates. Non-denaturing immunoprecipitates with immobilized to Affi-Prep Hz Hydrazide support M 110 antibodies were stained with different antibodies using a Multiscreen apparatus (Bio Rad). The results indicate that the protein with Mr ≈70 kD was recognized by all M 110, ANK 110, and LZ 110 antibodies, and this protein coimmunoprecipitates with myosin, MLCK, but not with DARPP or inhibitor-1 (they both are cross-reacted with DARPP antibodies (Chemicon).

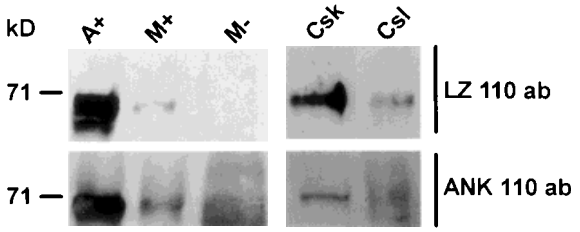

Fig. 5. Fractional distribution of M70 PPase 1 regulatory subunit. **Left**: Actin-enriched (A+), myosin-enriched (M+), and myosin-depleted fractions (M-) were prepared as we have previously described [Verin et al., 1995] and immunoblotted with LZ 110 (**upper blot**) or ANK110 antibodies (**lower blot**). Protein loading was equivalent to ≈25 µl of the initial homogenate. **Right**: Cytoskeletal (Csk, NP-40/ NaCl-insoluble) and cytosolic (Csl) BPAEC protein fractions prepared as we have previously described [Verin et al., 1998c] were probed with LZ 110 (**upper blot**) and ANK 110 antibodies (**lower blot**). The majority of M70 PPase 1 regulatory subunit was present in cytoskeletal EC or actin-enriched fractions, a significant amount was present in myosin-enriched fraction, and none was found in myosin-depleted fraction, suggesting the putative 70 kD RS1 is identified and selectively associates with contractile proteins in the EC cytoskeleton.

enriched fraction contains mainly actin, actin-associated cytoskeletal proteins, and significant amounts of myosin. The myosin-enriched fraction contains myosin and myosin-associated proteins, whereas the myosin-depleted fraction represents cytosolic proteins [Verin et al., 1995]. Western immunoblotting analysis of these EC fractions with LZ 110 and ANK 110 antibodies (Fig. 5), but not with control rabbit serum (data not shown) reveals the majority of M70 to be present in the actin-enriched fraction with detectable amounts in the myosin-enriched fraction but completely absent from the myosin-depleted fraction. Consistent with these results, M70 was abundant in the detergent-insoluble cytoskeletal fraction, but not in the detergent-soluble cytosolic fraction (Fig. 5). The results of these experiments again suggest a tight association between M70 and the acto-myosin cytoskeleton in endothelium.

DISCUSSION

Using an approach which primarily utilizes pharmacologic inhibitors, we have previously shown that PPase 1 is directly involved in endothelial cell contractility and barrier regulation [Verin et al., 1995]. In the present study, we have extended these findings and have shown that the myosin-associated PPase 1 in endothelium is likely to include M110 and M70 regulatory subunits and a CS1δ catalytic subunit. Molecular cloning has identified four PPase 1 catalytic subunit (CS1) isoforms: α, γ1, γ2, and δ [Sasaki et al., 1990] which are 90% identical and have similar functional characteristics [Sasaki et al., 1990; Zhang et al., 1993]. However, it is possible that the most variable C-terminal region of CS1 isoforms may determine its preferential association with specific regulatory subunits. For example, smooth muscle myosin PPase 1 regulatory subunits could preferentially form a complex with CS1δ, whereas other type 1 PPases exhibit less CS1 isoform-specific selectivity in vitro [Alessi et al., 1992; De Paoli-Roach et al., 1994]. Regulatory subunits specify both the function of the catalytic moiety as well as the distinct functional forms generated by combination of a similar catalytic component with different regulatory subunits, which target catalytic subunits to appropriate subcellular locations and substrates [Hubbard and Cohen, 1993]. For example, mammalian PPase 1 catalytic subunits can be directed to glycogen particles by a 124 kD subunit (R_{GL}) or can remain as a soluble cytoplasmic form by complexing with a 23 kD protein termed modulator subunit (inhibitor-2) [De Paoli-Roach et al., 1994]. PPase 2A is composed of a 36 kD catalytic subunit tightly complexed with a "constant" 65 kD regulatory subunit and a third or "variable" subunits, of varying size (54, 55, 72, 74, and 130 kDa), to form several trimeric holoenzymes [De Paoli-Roach et al., 1994; Zolnierowcz et al., 1994]. PPase 2B is a heterodimer of a 60 kD CaM-binding catalytic subunit (A subunit) and a 17 kD Ca^{2+}-binding B subunit, closely related to CaM [Kincaid, 1993] and only PPase 2C appears to be a 42 kD monosubunit enzyme [Shenolikar and Nairn, 1991]. Multiple attempts were made to purify the PPase complex responsible for myosin dephosphorylation from turkey gizzard extracts (SMP I-IV) [Pato et al., 1994], aortic smooth muscles, chicken gizzards, cardiac, and skeletal muscles [Alessi et al., 1992; Chen et al., 1994; Okubo et al., 1994; Shimizu et al., 1999; Mumby et al., 1987; Onishi et al., 1982; Dent et al., 1992], and have revealed a ≈37–40 kD catalytic subunit and a variety of regulatory subunits, which can be divided by molecular weight in four subgroups: 21 kD, 54–58 kD, 60–67 kD, and 130–133 kD. Recently 130 kD protein was cloned (M110 according to actual molecular weight) from several sources [Chen et al., 1994; Shimizu et al., 1994] and found to contain an ankyrin repeat region (amino acids 39–295 for M110 from chicken gizzard), which is important for determination of substrate specificity towards phosphorylase a and MLC [Hirano et al., 1997; Johnson et al., 1996] and may be involved in the interaction with the cytoskeleton [Shimizu et al., 1994]. The M110 regulatory subunit also exhibits an acidic cluster (326–372); two ionic clusters (719–755 and 814–848) and a Ser/Thr-rich region (770–793). Rat gizzard and human platelet M110, but not chicken gizzard M110, contain leucine zipper motifs located in C-terminus, which may be important for interaction with other proteins such as myosin [Chen et al., 1994; Muranyi et al., 1998]. The M21 subunit with unknown function, which is co-expressed in the complex with M110 shares significant homology with C-terminal part of M110 (leucine zipper motif) [Chen et al., 1994] but does not interact with CS1 although it does interact with the C-terminal part of M110 and myosin [Johnson et al., 1997]. Although M21 is always co-expressed in complex with M110 in smooth muscle [Shimizu et al., 1994], we could not detect M21 in endothelium by our LZ110 or ANK110 antibody. Similarly, M21 was not detected in cardiac muscle [Nishi et al., 1997] suggesting that M21 may be specifically expressed in smooth muscle. Recent studies indicate the 54–58 kD protein may represent an N-terminal proteolytic fragment of M110 which binds CS1 and myosin and stimulates dephosphorylation of MLC by CS1 [Okubo et al., 1994; Shimizu et al., 1994]. Targeting regulatory subunits of the myosin PPase in the 60–67 kD molecular weight range have been previously described in several tissues including smooth, cardiac, and skeletal muscle [Mumby et al., 1987; Onishi et al., 1982; Dent et al., 1992]. In the present study we describe a novel M70 regulatory subunit in bovine endothelium, with

immunohistochemical experiments indicates that M70, similar to M110, contains ankyrin repeats and leucine zipper motif regions, which are important for myosin PPase 1 regulation [Chen et al., 1994; Shimizu et al., 1994]. As both antibodies directed at the N-terminal (ANK 110) and C-terminal (LZ 110) regions of M110 recognize M70, it is highly unlikely that M70 represents a proteolytic fragment of M110. It is interesting to note that M110 isoforms described to date are very conserved in N-terminal part including the ankyrin repeat region, but differ markedly in the central portion of the molecule [Shimizu et al., 1994; Chen et al., 1994; Haystead et al., 1995]. We speculate that M70 may be an alternatively spliced product of M110 gene, which lacks a portion of central region/motif of the molecule. Several regulatory subunits have now been characterized which exist as alternatively spliced variants in the same cell. For example, cells expressing LIM kinase (LIMK-1), which is responsible for phosphorylation of cofilin in vivo [Arber et al., 1998], contain a spliced variant, LIMK1-short, that lacks a 20 amino acid stretch in the catalytic domain and has no kinase activity [Arber et al., 1998; Bernard et al., 1994]. LIMK1 and LIMK1-short can form homo- and heterodimers suggesting a mechanism by which LIMK1-short may negatively regulate the activity of LIMK1. The significance of co-expression of M70 and M110 is unknown, but potentially may be important for regulation of myosin-specific PPase activity in endothelium. Both M110 and M70 are present in MLCK and myosin immunoprecipitates as well as in whole BPAEC homogenates suggesting a tight association with myosin/MLCK functional complex. Our immunochemical data indicate that this complex also includes actin and the δ isoform of PPase 1 catalytic subunit, which is characteristic for the myosin PPase holoenzyme from smooth muscle [Shirazi et al., 1994]. Recently, Sobieszek et al. [1997a,b] purified and characterized a myofibrillar form of PPase from turkey gizzard, which includes a 67 kD targeting subunit and 37 kD catalytic subunit. The structural features and the relationship to M110 or M70 to M67 are unknown, but similar to our results in endothelium, M67 was noted to be tightly bound to MLCK in a functional complex [Sobieszek et al., 1997a,b]. According to our fractionation experiments, M70 has primarily a cytoskeletal localization. Overlay assay analysis revealed an existence of four putative regulatory subunits (PPbp216, PPbp175, PPbp134, and PPbp75) of human brain PPase 1, which likely target PPase 1 to the cytoskeleton [Colbran et al., 1997]. While it is interesting to speculate that M70 may be necessary to link PPase/myosin/MLCK complex to the endothelial cytoskeleton, further studies are required to more clearly define the role of M70 in the regulation of EC myosin-specific PPase activity.

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