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Purification of dystrophin-related protein (utrophin) from lung and its identification in pulmonary artery endothelial cells

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Dystrophin-related protein (DRP or utrophin) is an autosomal homologue of dystrophin, a membrane cytoskeletal protein encoded by the Duchenne muscular dystrophy gene. In contrast to dystrophin which is predominantly expressed in muscle, DRP is expressed in various tissues. Here we report the purification and biochemical characterization of DRP from lung which shows the highest levels of DRP expression among adult tissues. DRP was purified from the high alkaline extract of lung membranes using heparin-agarose column chromatography followed by anti-DRP immunoaffinity column chromatography. DRP was expressed in the cultured pulmonary artery endothelial cells. Expression of DRP in endothelial cells could explain its abundant expression in lung. In analogy to dystrophin of muscle cells, DRP could be playing an important role in the mechanical stress mechanisms of endothelial cells.

Dystrophin-related protein; Utrophin; Dystrophin; Membrane cytoskeleton; Lung; Endothelial cell

1. INTRODUCTION

Duchenne muscular dystrophy (DMD) patients and *mdx* mice are characterized by the absence of dystrophin, a large membrane cytoskeletal protein encoded by the DMD gene on the X chromosome [1,2]. Dystrophin is associated with a large oligomeric complex of sarcolemmal glycoproteins, including dystroglycan which provides a linkage to the extracellular matrix component, laminin [3–10]. In DMD patients and *mdx* mice, the absence of dystrophin leads to a great reduction in all of the dystrophin-associated proteins in the sarcolemma, thus causing the disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix [4,9–11].

Recently, a transcript of similar size and high homology to dystrophin was cloned from skeletal muscle, and the gene was mapped to chromosome 6 in human and chromosome 10 in mouse [12,13]. Immunoblot analysis of skeletal muscle homogenates has shown that the protein product of this gene, dystrophin-related protein (DRP or utrophin), is similar in size to dystrophin [14–21]. In adult skeletal muscle, DRP is localized to the neuromuscular junction [15,17–20,22], where it is associated with a large oligomeric complex of sarcolemmal glycoproteins which are identical with or antigenically similar to the dystrophin-associated proteins [22]. A possibility that the upregulation of DRP could have compensatory effects for dystrophin deficiency was raised [18–20,22,23], based on the findings that DRP is overexpressed and the dystrophin/DRP-associated proteins are preserved in the small-caliber skeletal and cardiac muscles of *mdx* mice which show minimal pathological changes [22].

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The homology of the amino acid sequence between DRP and dystrophin is high for the two domains which have important physiological functions [2,12,24]: (1) the amino-terminal domain which contains the actin-binding site [2,25–27]; and (2) the cysteine-rich and carboxyl-terminal domains which are involved in the interaction with the sarcolemmal glycoprotein complex in skeletal muscle [28,29]. On the other hand, the sequence homology between these two proteins is relatively low for the rod domain which consists of repeats of triple helix [2,24]. The tissue distribution of DRP and dystrophin is also different. Dystrophin is predominantly expressed in muscle and neuronal cells, while DRP is expressed in various tissues including brain, choroid plexus, muscle, lung, liver, kidney, testis and placenta, and in various cultured cell lines [14,16,19,21,30], suggesting distinct biochemical characteristics and functions of these two homologous proteins. However, the biochemical characteristics and function of DRP in non-muscle tissues remain unknown.

In the present study, we report for the first time the purification of DRP from lung, which shows the highest levels of DRP expression among adult tissues. We also demonstrate the expression of DRP in cultured pulmonary artery endothelial cells. Our results suggest that the

expression of DRP in endothelial cells could explain its abundant expression in lung.

2. EXPERIMENTAL

2.1. *Purification of DRP from lung and skeletal muscle*

Crude membranes were prepared from lung, skeletal muscle (back and hindlimb muscles), cardiac muscle, small intestine, brain, liver, kidney and spleen of adult rabbit as described previously [31]. KCl-washed heavy microsomes were prepared from skeletal muscle of adult rabbit as described previously [3,4,6].

Crude lung membranes or KCl-washed heavy microsomes of skeletal muscle (1,500 mg) were suspended in 50 mM Tris-HCl, pH 7.4, containing 0.1 mM PMSF and 0.75 mM benzamidine (buffer A) at the protein concentration of 5 mg/ml. The suspension was titrated to pH 11 by slowly adding 1 N NaOH, extracted by gentle stirring for 1 h at room temperature and then centrifuged in a Beckman Type 45 Ti rotor at 140,000 × *g* for 30 min at 25°C. After 4 M NaCl was added to the supernatant to a final concentration of 0.15 M NaCl, the supernatant was titrated to pH 7.4 by slowly adding 1 N HCl, cooled in ice immediately, titrated to pH 7.4 again at 4°C and centrifuged in a Beckman Type 45 Ti rotor at 140,000 × *g* for 30 min at 4°C. The supernatants were circulated overnight on a 40 ml Heparin-agarose column pre-equilibrated with buffer A containing 0.15 M NaCl. After extensive washing with buffer A containing 0.15 M NaCl, the column was eluted with 150 ml of buffer A containing 0.6 M NaCl.

Anti-DRP immunoaffinity column was prepared by cross-linking antibody against DRP with protein A-Sepharose beads as described previously [22]. The 0.6 M NaCl eluates of the heparin-agarose column chromatography were diluted 1.2 times with buffer A and circulated overnight on a 20 ml protein A-Sepharose column pre-equilibrated with buffer A containing 0.5 M NaCl. The voids of the protein A-Sepharose column chromatography were circulated overnight on a 5 ml anti-DRP immunoaffinity column pre-equilibrated with buffer A containing 0.5 M NaCl. After extensive washing with buffer A containing 0.5 M NaCl, the column was eluted with 20 ml of 0.1 M glycine-HCl, pH 3.0, 0.1 mM PMSF, 0.75 mM benzamidine and 0.5 M NaCl followed by 40 ml of buffer A containing 0.5 M NaCl. Four-ml fractions were collected and neutralized with 2 M Tris-HCl, pH 8.0, immediately.

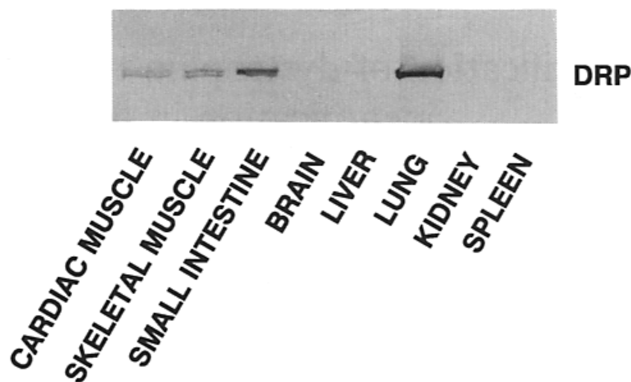

Fig. 1. Distribution of DRP in tissues of adult rabbit. Membranes (250 μg) from cardiac muscle, skeletal muscle, small intestine, brain, liver, lung, kidney and spleen were fractionated by 3–12% SDS-PAGE, transferred to nitrocellulose membranes and immunostained with antibody against DRP.

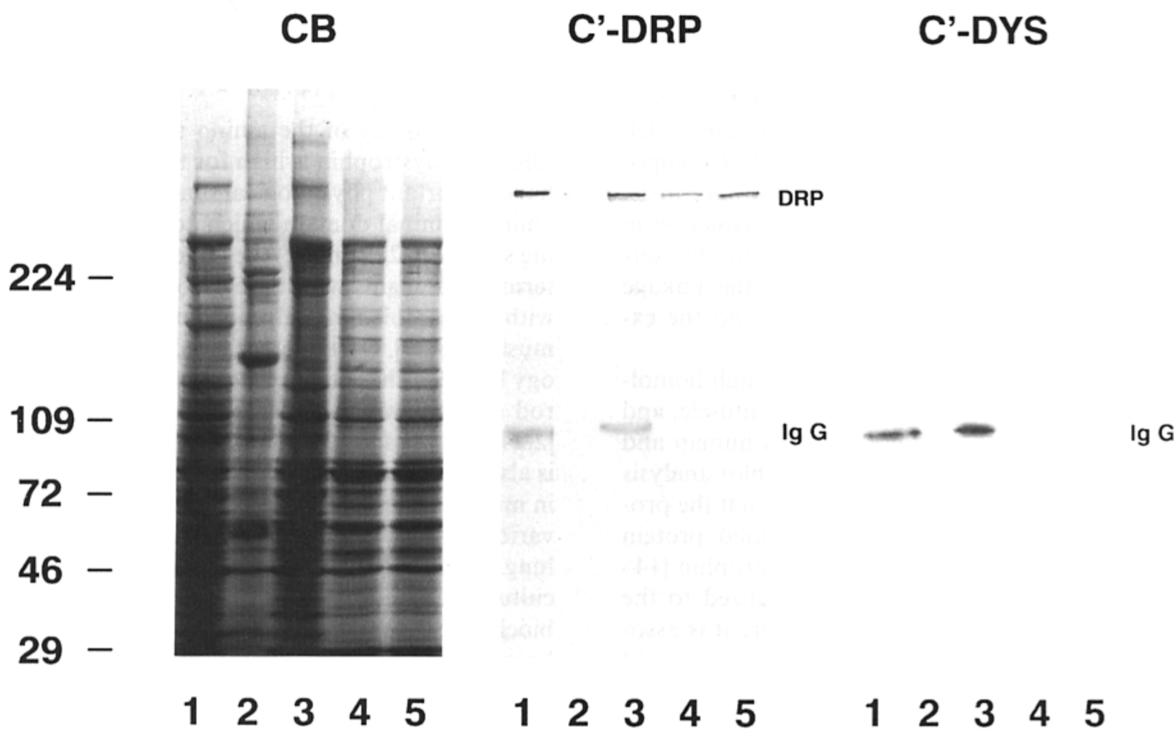

Fig. 2. Heparin-agarose column chromatography of proteins extracted from rabbit lung membranes by high-alkaline treatment. Crude membranes (lane 1), the combined pellets of two centrifugations after alkaline extraction (lane 2), the supernatant of the second centrifugation (lane 3), the 0.6 M NaCl eluate of the heparin-agarose column chromatography (lane 4) and the void of the protein A-Sepharose column chromatography (lane 5) were fractionated by 3–12% SDS-PAGE. Shown are the Coomassie blue-stained gel (CB) and identical nitrocellulose transfers immunostained with antibody against DRP (C′-DRP) or dystrophin (C′-DYS). Molecular weight standards (× 10^{-3}) are shown on the left. Immunoglobulin G (IgG), which was present in the lung membranes and was immunostained by peroxidase-labeled anti-rabbit IgG secondary antibody, did not bind to the heparin-agarose column in the presence of 0.15 M NaCl.

2.2. *Identification of DRP in the cultured pulmonary artery endothelial cells*

Porcine pulmonary arteries were obtained immediately after slaughter at a local abattoir. Resected ends of the arteries were clamped, and the artery segment briefly immersed in 70% ethanol then immediately rinsed in M199 with penicillin (100 units/ml) and streptomycin (100 μg/ml) (1XP + S). Arteries were then unclamped and placed in sterile M199 with 1XP + S. In the laboratory, the arteries were opened longitudinally and the lumen gently scraped with a cotton tipped applicator. Cells were released into 35 mm diameter tissue culture dishes with M199 supplemented with Basal Medium Eagle vitamins and amino acids, 1XP+S, 10% fetal bovine serum and then incubated at 37°C in 5% CO_2 and 95% air. Cultures were demonstrated to be endothelial by morphology and by the uptake of acetylated low density lipoprotein [32]. Cells were subcultured 3:1 twice weekly using 0.25% trypsin:0.1% EDTA, and cultures from passages 4 through 10 were used.

Cultured endothelial cells were extracted on the dish for 10 min at room temperature with extraction buffer consisting of 10 mM PIPES-NaOH, pH 7.4, 100 mM sodium pyrophosphate, 1% digitonin, 0.5 M NaCl, 0.5 M sucrose, 1 mM EGTA, 2 mM $MgCl_2$, 5 mM aminocaproic acid, 0.1 mM PMSF, 5 mM benzamidine, 100 μM leupeptin and 80 μg/ml of aprotinin. The extract (1 ml) was incubated at 4°C overnight with 100 μl of the anti-DRP immunoaffinity beads which were pre-equilibrated with extraction buffer. After centrifugation, the supernatants were decanted and analyzed by immunoblotting.

2.3. *Antibodies*

Affinity-purified rabbit polyclonal antibody against the last 12 amino acids of the C-terminus of DRP, rabbit polyclonal antibody against the last 10 amino acids of the C-terminus of dystrophin and two monoclonal antibodies against dystrophin, $VIA4_2$ and XIXC2, were characterized previously [4,6–11,17,22,33]. 3–12% SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed as described previously [3,4].

3. RESULTS AND DISCUSSION

Immunoblot analysis of membranes from various tissues of adult rabbit demonstrated that the highest levels of DRP expression were in lung, although it was also detected in small intestine, skeletal muscle, cardiac muscle, liver, kidney and brain (Fig. 1). We have previously demonstrated that the dystrophin–glycoprotein complex is dissociated at pH 11 [6]. Further investigation has revealed that dystrophin, a membrane cytoskeletal protein, is extracted from skeletal muscle membranes by the pH 11 treatment, while the dystrophin-associated glycoproteins remain in the membranes [34]. We decided to perform the pH 11 extraction of the membranes from lung and skeletal muscle as the initial step for the purification of DRP. DRP was extracted from both crude lung membranes and KCl-washed heavy microsomes of skeletal muscle by this procedure (Figs. 2 and 3), suggesting that DRP may also be a membrane cytoskeletal protein like dystrophin.

After the overnight circulation on the heparin-agarose column in the presence of 0.15 M NaCl and the extensive wash of the column, the bound proteins were eluted with a buffer containing 0.6 M NaCl. DRP eluted completely in the 0.6 M NaCl buffer (Figs. 2 and 3). Dystrophin was not detected in lung, while dystrophin co-purified with DRP from skeletal muscle during these procedures (Figs. 2 and 3). These results are consistent

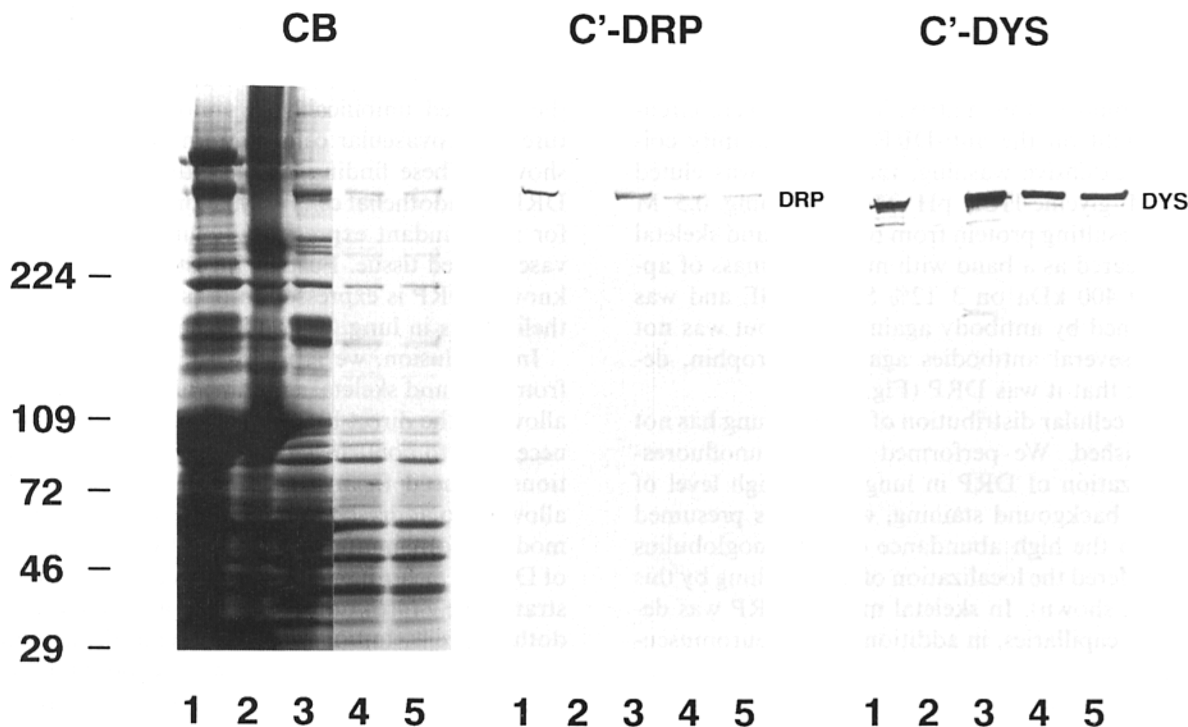

Fig. 3. Heparin-agarose column chromatography of proteins extracted from rabbit skeletal muscle membranes by high-alkaline treatment. KCl-washed heavy microsomes (lane 1), the combined pellets of two centrifugations after alkaline extraction (lane 2), the supernatant of the second centrifugation (lane 3), the 0.6 M NaCl eluate of the heparin-agarose column chromatography (lane 4) and the void of the protein A-Sepharose column chromatography (lane 5) were fractionated by 3–12% SDS-PAGE. Shown are the Coomassie blue-stained gel (CB) and identical nitrocellulose transfers immunostained with antibody against DRP (C′-DRP) or dystrophin (C′-DYS).

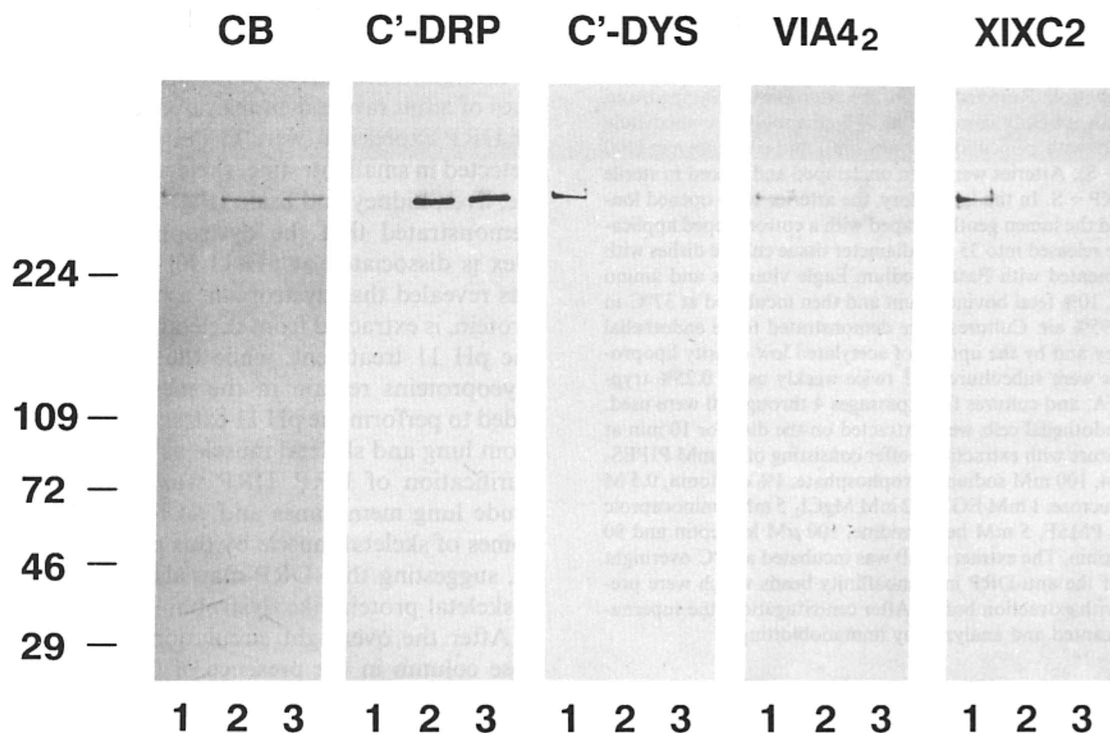

Fig. 4. Purification of DRP using the anti-DRP immunoaffinity column chromatography. The peak fractions of the anti-DRP immunoaffinity column eluates from skeletal muscle (lane 2) and lung (lane 3) were fractionated by 3–12% SDS-PAGE together with dystrophin isolated from rabbit skeletal muscle as described previously (lane 1) [6]. CB indicates the gel stained with Coomassie blue. Identical nitrocellulose transfers were immunostained with antibody against DRP (C′-DRP) or antibodies against dystrophin (C′-DYS, $VIA4_2$ and XIXC2).

with the high homology between DRP and dystrophin.

The eluates of the heparin-agarose column chromatography from lung and skeletal muscle were circulated on the protein A-Sepharose column to remove proteins which may bind to this matrix. The voids were circulated overnight on the anti-DRP immunoaffinity column. After extensive washing, the column was eluted with 0.1 M glycine-HCl, pH 3.0, containing 0.5 M NaCl. The resulting protein from both lung and skeletal muscle appeared as a band with molecular mass of approximately 400 kDa on 3–12% SDS-PAGE and was immunostained by antibody against DRP but was not stained by several antibodies against dystrophin, demonstrating that it was DRP (Fig. 4).

So far the cellular distribution of DRP in lung has not been established. We performed the immunofluorescence localization of DRP in lung but a high level of non-specific backgound staining, which was presumed to be due to the high abundance of immunoglobulins (Fig. 2), hindered the localization of DRP in lung by this method (not shown). In skeletal muscle, DRP was detected in the capillaries, in addition to the neuromuscular junctions (not shown). This suggested that DRP could be expressed in the endothelial cells which are abundant in lung. We investigated this possibility using cultured pulmonary artery endothelial cells. The digitonin-extracts of the cultured pulmonary artery endothelial cells were subjected to immunoprecipitation by the anti-DRP immunoaffinity beads. Immunoblot analysis demonstrated that DRP was expressed in pulmonary artery endothelial cells (Fig. 5). DRP in the digitonin-extracts was precipitated completely by the anti-DRP antibody (Fig. 5). DRP was also detected in the cultured umbilical vein endothelial cells and cultured microvascular cells by immunoblot analysis (not shown). These findings suggest that the expression of DRP in endothelial cells may be one of the explanations for its abundant expression in lung, which is a highly vascularized tissue. Further investigation is required to know if DRP is expressed in cells other than the endothelial cells in lung.

In conclusion, we reported the purification of DRP from lung and skeletal muscle. The present work would allow for the direct biochemical study of DRP which is necessary to confirm structural and functional predictions deduced from the cDNA sequence [12,24], and allow us to address the possibility of post-translational modifications important to the physiological function of DRP in non-muscle tissues and cells. We also demonstrated the expression of DRP in pulmonary artery endothelial cells. Interestingly, both endothelial and muscle cells are highly distensible when exposed to mechanical stress. As a membrane cytoskeletal protein, DRP could be playing an important role in the mechanical stress mechanisms of endothelial cells [35], in analogy to dystrophin of muscle cells [8,10]. The upregulation of DRP in dystrophin-deficient muscle was recently proposed as one of the potential therapies for DMD [18–

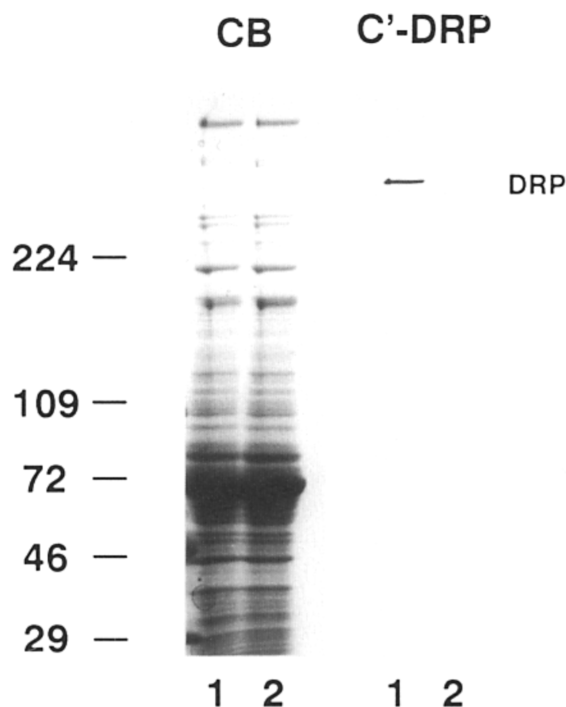

Fig. 5. Immunoprecipitation of DRP from cultured pulmonary artery endothelial cells. The voids of digitonin-extracts after incubation with the anti-DRP immunoaffinity beads (lane 2) or control beads (lane 1) are shown. Shown are the gel stained with Coomassie blue (CB) and the nitrocellulose transfers immunostained with antibody against DRP (C′-DRP).

20,22,23]. Identification of DRP in endothelial cells raises a serious question concerning this possibility. For such a therapy to be successful, the upregulation of DRP should be specific to muscle cells and should not involve endothelial cells, since the overexpression of DRP in endothelial cells could affect blood circulation. If different promoters for muscle and endothelial cell DRP are identified, a muscle-specific promoter could be utilized for the upregulation of DRP in muscle cells.

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