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A REGULATORY SUBUNIT OF SMOOTH MUSCLE MYOSIN BOUND PHOSPHATASE

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Summary: The relationship between two putative myosin-binding subunits of smooth muscle myosin phosphatase was investigated. A monoclonal antibody (MoAb) to the 58 kD component of smooth muscle myosin-bound phosphatase (MBP) cross-reacted with a 130 kD protein in extracts of fresh chicken gizzards. The MoAb in combination with protein A immunoprecipitated from gizzard extracts a complex of the 130 kD protein plus the 38 kD catalytic subunit of the type 1δ protein phosphatase. It is proposed that the 130 kD component is a native subunit of MBP and that the 58 kD protein is its proteolytic degradation product. The distribution of the 130 kD component in chicken tissues was screened using the MoAb. An immunoreactive band of appropriate mass was detected in all tissues except liver and skeletal muscle. Higher concentrations of the 130 kD component were evident in the smooth muscle samples.

Contractile activity in smooth muscle is determined by the level of myosin phosphorylation (1) and this reflects the activities of myosin light chain kinase (MLCK) and the myosin phosphatase. For a given [Ca^{2+}] it was assumed (1) that the extent of myosin phosphorylation would be fixed. However, recent experiments with intact smooth muscle have shown that the phosphorylation-Ca^{2+} relationship is variable and for example is sensitive to the method of stimulation (2-4). Thus the activity of one of the two key regulatory enzymes can be altered and one suggestion is that on agonist stimulation the phosphatase is inhibited (5). The underlying mechanism for this is not known and a major difficulty is that the relevant phosphatase subunits are not defined.

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Abbreviations: MBP, myosin-bound phosphatase; PP1δ, type 1δ protein phosphatase; MoAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(b-aminoethyl ether)-Çò-tetraacetic acid; DTT, dithiothreitol; aPMSF, (*p*-amidinophenyl) methanesulfonyl fluoride; MLCK, myosin light chain kinase.

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Despite several reports on the isolation of myosin phosphatases (review 6) only two emphasize possible regulatory subunits. In one of these (7) a trimeric phosphatase, composed of 130, 37 and 20 kD subunits was isolated from chicken gizzard. It was suggested, based on activity measurements, that the 130 and 20 kD components acted as targeting subunits to myosin and thus could serve in a regulatory role. Whether both subunits were necessary was not determined. In the second report the interaction with myosin was demonstrated directly but involved a 58 kD subunit as part of a dimeric complex with the phosphatase type 1 (PP-1) δ catalytic subunit (38 kD). This preparation was termed the myosin-bound phosphatase (MBP, 6). But it is not known if the 58 kD (6) and the 130 kD (7) subunits are related.

A monoclonal antibody (MoAb) has been raised to the 58 kD subunit and is used to determine if the 58 and 130 kD subunits are related. The data presented in this article show that the 58 kD and the larger subunit are related. In addition, application of the MoAb allowed us to screen several tissues for the presence of the MBP subunit. Type 1 phosphatases are widely distributed (8) and it is assumed that each of the 4 isozymes (9) has multiple roles. There is, however, no information available on whether the subunit associated with smooth muscle myosin has a broader distribution and thus a more general function.

MATERIALS AND METHODS

Purification of proteins: Smooth muscle myosin-bound phosphatase (MBP) was purified to homogeneity from chicken gizzard by the method of Okubo *et al.* (6). Smooth muscle myosin (10) and MLCK (11) were purified as described previously.

Antibody production: Polyclonal antibodies were raised in rabbits against gizzard MLCK (12) and the catalytic subunit of PP 1δ (13). The MoAb against the 58 kD myosin-binding subunit was prepared according to the methods of Kennett (14) and Sato *et al.* (15) with some modifications (16). The MoAb was purified from the supernatant of a culture grown in protein free Hybridoma Media-II (Gibco BRL, Gaithersburg, MD, USA) by DEAE-Cellulofine (Seikagaku Kogyo, Tokyo, Japan) by the procedure of Goding (17). The MoAb was an IgG_{2b}.

Procedures: SDS-polyacrylamide gel electrophoresis in 7.5 to 20 % gradient gels (18) and Western blots (19) were carried out as described previously. The immunocomplex was detected on photographic film by H_2O_2/luminol chemiluminescence (Amersham, Bucks., England). Immunoprecipitation of phosphatase was carried out by the method of Kawamoto and Adelstein (20) with the following modifications. Frozen chicken gizzards (10 g) were homogenized with a Polytron in 5 volumes of 400 mM NaCl, 50 mM Tris-HCl (pH 7.2), 2 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 μM (p-amidinophenyl) methanesulfonyl fluoride hydrochloride (aPMSF), 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor, and centrifuged at 100,000xg for 1 hr To 20 μl of supernatant was added 20 μl purified MoAb (0.15mg/ml). After 2 hrs incubation at 4 °C, 30 μl protein A Sepharose CL-4B (0.1 mg/ml) suspended in 30 mM Tris-HCl (pH 7.2) and 16.7 μl of 3M NaCl were added to a final volume of 100 μl. After an additional 1 hr incubation at 4 °C, the mixture was centrifuged at 12,000 rpm for 3 min at 4 °C in an Eppendorf microcentrifuge. Sediments were washed three times with 10 volumes of 500 mM NaCl, 20 mM Tris-HCl (pH 7.5) and 0.05% Tween 20, and dissolved in SDS-sample buffer containing 100 mM Tris-HCl (pH 6.8), 4 % SDS, 10 % glycerol, 0.02 % NaN_3 and 0.02 % brilliant phenol blue. For the immunoblot analysis of extracts from various tissues, using the MoAb, each tissue was freshly removed from the chicken and rapidly frozen in liquid nitrogen, homogenized with a Polytron in several volumes of 500 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM benzamidine, 1 μM aPMSF, 10 μg/ml leupeptin and 0.1mM diisopropyl fluorophosphate. After centrifugation at 20,000xg for 30 min, the supernatant was subjected to SDS-PAGE followed by immunoblotting. Protein concentrations were estimated by the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL, USA).

Materials: Protein A Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden). Affinity purified goat anti-rabbit IgG and anti-mouse IgG coupled to peroxidase were purchase from Bio-Rad (Richmond, CA, USA) and Cappel (West Chester, PA, USA), respectively. All other chemicals were of the highest grade commercially available.

RESULTS AND DISCUSSION

The MoAb reacted with the 58 kD subunit of purified smooth muscle MBP as shown in Fig. 1 (lane 1). However, the cross-reacting species in gizzard extract and in gizzard myosin was a 130 kD component (Fig. 1, lane 2 and 3, respectively). In the latter two Western blots a 58 kD was not detected. (Note that the respective protein stains for the Western blots are shown in Fig. 1D). Brief tryptic proteolysis of gizzard myosin converted the 130 kD band to the 58 kD and a minor 33 kD band (Fig. 1, lane 4). The polyclonal antibody specific to the PP 1δ isoform was used to screen these samples. As shown in Fig. 1B the catalytic subunit of the δ isoform was detected at 38 kD in purified MBP (lane 6), in gizzard extract (lane 7) and in gizzard myosin (lane 8). The mobilities on SDS-PAGE of MLCK and the 130 kD phosphatase subunit are similar. In addition, it is known that MLCK binds to myosin (21). Thus the question was raised whether the 130 kD phosphatase subunit is MLCK? This possibility was eliminated using the MoAb and a polyclonal antibody to MLCK. The MoAb did not cross react with isolated MLCK (Fig. 1, lane 5) and the polyclonal antibody did not cross react with purified MBP (Fig. 1, lane 9). (It should be pointed out that the polyclonal antibody to MLCK recognizes fragments of MLCK, but did not cross react with the 58 kD subunit of MBP). These results indicate that the 58 kD subunit is a proteolytic fragment of the 130 kD subunit and that the latter is not MLCK. In addition, it is confirmed (6) that the catalytic subunit associated with the 58 and 130 kD subunit is the δ isoform of PP 1.

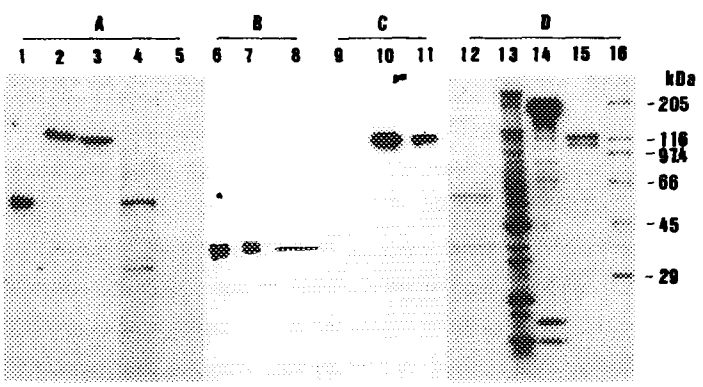

Fig. 1. Immunoblot analysis using anti-58 kD MoAb (MoAb-58), anti-PP 1δ antibody and anti-MLCK antibody. After SDS-PAGE, proteins were transferred to nitrocellulose membranes and either subjected to MoAb-58 (A), anti-PP 1δ antibody (B) or anti-MLCK antibody (C) followed by peroxidase staining of second antibody, or by protein staining with naphthol blue black (D) as described in "MATERIALS AND METHODS". Tryptic proteolysis of myosin was performed as follows: myosin (300 μg) in 30 mM Tris-HCl (pH 7.5), 0.3 M NaCl was incubated for 10 min at 25 °C with trypsin (26 pg) and the reaction was stopped by the addition of 1 μl of 0.1 M diisopropyl fluorophosphate (final 1 mM). Purified MBP (3 μg) from chicken gizzard in lanes 1, 6, 9 and 12; gizzard extract (60 μg) in lanes 2, 7, 10 and 13; 170 μg of purified gizzard myosin in lanes 3 and 8, and 15 μg in lane 14; trypsin-digested myosin (150 μg) in lane 4; 0.5 μg of purified MLCK in lanes 5, 0.1 μg in lane 11 and 3 μg in lane 15. Molecular weight standards in lane 16.

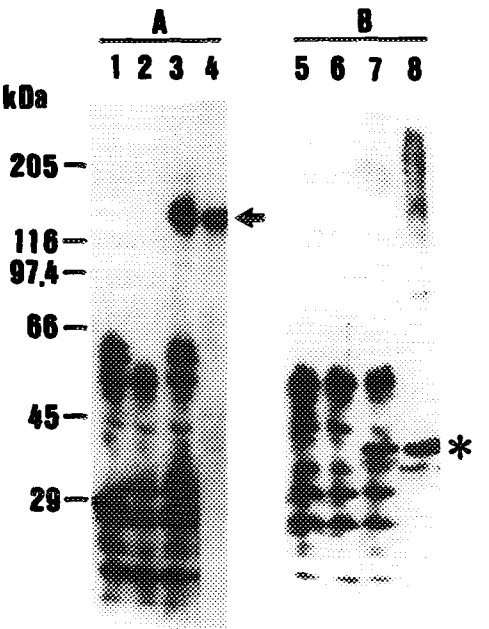

Fig. 2. Immunoprecipitation of MBP from a gizzard extract. Various fractions were subjected to SDS-PAGE, transferred to nitrocellulose membranes and immunoblots carried out with anti-58 kD MoAb (MoAb-58) (A) and anti-PP1δ antibody (B). Fractions analyzed were: an immunoprecipitate obtained from a gizzard extract with the MoAb-58 and protein A complex, lanes 3 and 7; precipitate of protein A and MoAb-58 minus gizzard extract (control), lanes 1 and 5; protein A alone (control), lanes 2 and 6; and blots of the gizzard extract, lanes 4 and 8. The arrow indicates the 130 kD subunit and the asterisk the 38 kD catalytic subunit.

To determine if the 130 kD component is associated in vivo with the catalytic subunit of PP 1 an immunoprecipitate was formed from gizzard extract using the MoAb-Protein A complex. The procedure was to submit the Protein A precipitates to SDS-PAGE followed by immunoblotting with either the MoAb (Fig. 2, lanes 1-3) or the polyclonal antibody to PP 1δ (lanes 5-7). Since both of the second antibodies used (i.e. anti-mouse and anti-rabbit) cross reacted with components of the Protein A-MoAb complex (i.e. in the absence of the gizzard extract) several controls were necessary. As shown in Fig. 2 (lanes 2 and 6) Protein A alone gave complicated patterns. These were not altered by the addition of gizzard extract (data not shown). For the Protein A-MoAb complex, additional components at 60, 56 and 27 kD are indicated with the anti-mouse second antibody (lane 1) and these reflect the heavy chain doublet and light chain of the MoAb, respectively. However, the immunoprecipitates gave distinct patterns. As shown by screening with the anti-58 kD MoAb a component of 130 kD was precipitated from the gizzard extract (Fig. 2, lane 3). The same immunoprecipitate when screened with the anti-PP 1δ antibody showed a component of about 38 kD (Fig. 2, lane 7). These bands correspond to the 130 kD component (detected with the MoAb) and the 38 kD component (detected with the anti-PP 1δ antibody) in gizzard extract, as shown in Fig. 2, lanes 4 and 8, respectively. These results show that both the 130 and 38 kD subunit are precipitated by the Protein A-MoAb complex and thus indicate that the two components are associated even at the relatively high ionic strength (0.5 M NaCl) of the gizzard extract.

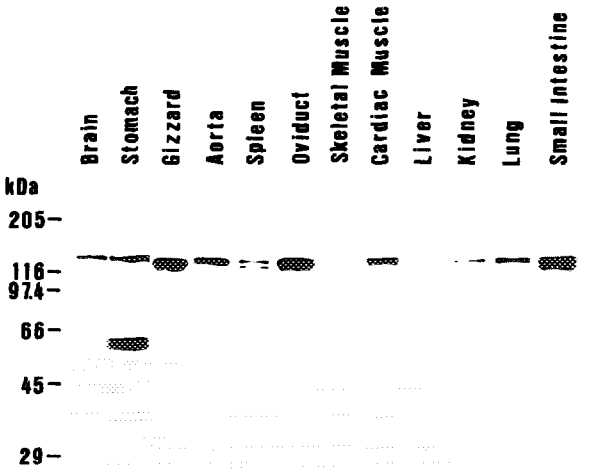

Fig. 3. Tissue distribution of immunoreactive components with anti-58 kD MoAb. The extracts (30 μg) of each tissue were applied to SDS-PAGE followed by immunoblot using the MoAb as described in "MATERIALS AND METHODS".

The distribution of the 130 kD subunit was investigated in various chicken tissues using the anti-58 kD MoAb. Reactive components were not detected in tissues from other species. As shown in Fig. 3, immunoreactive proteins of appropriate mass were detected in most tissues, except skeletal muscle and liver. They appeared more abundant in the smooth muscle tissues, i.e. gizzard, aorta, stomach, small intestine and oviduct, compared to brain, spleen, kidney and lung. Some differences in apparent molecular weight were observed. In gizzard the major band was approximately 130 kD although a minor band of 133 kD also was detected. A doublet of about 137 and 125 kD was observed in spleen, oviduct and small intestine and in cardiac muscle a doublet of slightly higher molecular weights (143 and 137 kD) was found. The two bands seen in stomach probably represent conversion of the 137 kD component to the 58 kD band via proteolysis, since the intrinsic protease activity of this tissue is high. A single immunoreactive band of about 137 kD was found in brain, aorta, kidney and lung. The presence of these distinct immunoreactive components could indicate that either a high molecular weight parent molecule is clipped by proteolysis or that different isoforms of this subunit exist.

The widespread distribution of the larger phosphatase subunit is interesting and clearly demonstrates that this phosphatase system is not confined to smooth muscle. If it is assumed that the "130" kD subunit binds to the PP 1 catalytic subunit and also binds to myosin then it would be involved in a variety of myosin-linked processes and these may include non-contractile mechanisms, e.g. in the non-muscle tissues. It is surprising that the "130" kD subunit was detected in cardiac but not in skeletal muscle, since the contractile mechanisms of the two tissues are similar. Myosin phosphorylation does not play a dominant role in striated muscle. Recently it has been shown (22) that the myosin-targeting subunit in skeletal muscle was distinct from the smooth muscle enzyme.

In this study it is shown that the 58 kD subunit of MBP (6) is derived via proteolysis from the 130 kD subunit (7). Although it is a fragment of the native protein, it retains several important

properties. Notably it binds to myosin and also binds to the PP 1 catalytic subunit. The differences in function between the 130 kD parent molecule and the 58 kD fragment are not known. It is possible that the parent molecule also is involved in binding the 20 kD subunit (7) since this component was not present in the preparation described by Okubo *et al.* (6), i.e. the complex of 58 and 38 kD subunits. Studies are underway to define the functions of different regions of the 130 kD subunit.

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