Inhibition of 20-kDa myosin light chain exchange by monoclonal antibodies against 17-kDa myosin light chain

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Abstract Two anti-17,000 Da myosin light chain (LC17) monoclonal antibodies (MM2 and MM10), which increase the actin-activated Mg^{2+}-ATPase activity of dephosphorylated smooth muscle myosin, inhibited the exchange of the 20,000 Da regulatory light chain of myosin (LC20). MM2, which shows higher potency of activation of ATPase activity, inhibited the exchange more extensively than MM10, suggesting that there is a correlation between the activation of ATPase activity and the inhibition of the LC20 exchange. The inhibition of the exchange was observed for intact myosin and heavy meromyosin but not subfragment 1, suggesting that the heavy chain at the head–rod junction is involved in the inhibition of LC20 exchange by anti-LC17 antibodies. Alternatively, the interaction between the two heads of the myosin molecule may influence the inhibition of LC20 exchange. These results suggest that LC20 interacts with both LC17 and the heavy chain, and the interaction between LC20 and LC17 is involved in the activation of actin-activated ATPase activity of smooth muscle myosin.

Key words: Smooth muscle myosin; 20-kDa myosin light chain; Monoclonal antibody

1. Introduction

The motor activity of actomyosin from vertebrate smooth muscle and non-muscle cells is regulated by the phosphorylation of the 20,000 Da (20-kDa) light chain (LC20) of myosin [1,2]. The phosphorylation site responsible for the regulation is Ser-19 of LC20 which is catalyzed by Ca^{2+}/calmodulin dependent myosin light chain kinase (MLCK). Recent analysis of the crystal structure of skeletal muscle myosin subfragment-1 (S-1) [3] and the regulatory domain of scallop myosin [4] revealed that this class of light chain is associated with myosin heavy chain at the head–rod junction. While the molecular mechanism by which the phosphorylation of LC20 regulates actomyosin motor activity is not understood, it is plausible that the conformational change of LC20 induced by phosphorylation is transmitted to the heavy chain either directly or via the 17,000 Da (17-kDa) light chain (LC17) which ultimately affects the effector sites of myosin located at the top of the head. The change in LC20 conformation could first be transmitted to the heavy chain at the head–rod junction.

We have hypothesized [5–9] that phosphorylation of LC20 induces the change in the conformation at the head–rod junction of myosin and this is involved in the regulation mechanism of smooth muscle actomyosin motor activity because: (i) S-1, but not heavy meromyosin (HMM), containing intact light chains fails to show phosphorylation dependence on its actin-activated ATPase, suggesting that the head–rod hinge region plays a role in the regulation [5]; (ii) LC20 phosphorylation changes the conformation at the head–rod junction which is indicated by several experimental observations, i.e. increase in the digestibility at the head–rod junction [6], increase in the flexibility at the hinge region [7], and change in the head orientation observed by electron microscopy [8,9].

While the regulatory function of LC20 in the smooth muscle actomyosin system is well documented, the function of LC17 is not clear. Previously, we reported that a monoclonal antibody which recognizes LC17 can activate the actin-activated ATPase activity of myosin without LC20 phosphorylation [10]. This suggested that LC17 might be involved in the regulatory mechanism of phosphorylation-induced activation of actomyosin. Recent structural analysis of the scallop myosin regulatory domain revealed [4] that the N-terminal domain of the essential light chain (corresponding to LC17) interacts with the regulatory light chain (corresponding to LC20) which stabilizes Ca^{2+} binding to the N-terminal domain of the essential light chain so as to regulate actomyosin ATPase activity. On the other hand, it is not known whether or not LC20 and LC17 interact with each other and thus contribute to the transmission of phosphorylation-induced signal to the effector sites.

In the present study, we examined the interaction between LC20, LC17 and the heavy chain of smooth muscle myosin using specific monoclonal antibodies as probes.

2. Material and methods

2.1. Materials

Smooth muscle myosin and myosin light chain kinase were prepared from turkey gizzard [11]. Heavy meromyosin (HMM) and subfragment-1 (S-1) were prepared by digestion of myosin by *Staphylococcus aureus* protease (SAP) as described previously [5]. Phosphorylated LC20 was prepared as described previously [12]. Calmodulin was prepared from bull testes according to Walsh et al. [13].

2.2. Production of monoclonal antibodies against smooth muscle myosin

Balb/c mice were immunized with intact smooth muscle myosin. Screening and purification of antibodies were performed as described elsewhere [10]. IgG monoclonal antibodies (MM6, MM9 and MM10) were purified using a protein A column, and IgM monoclonal antibody (MM2) was purified using DEAE-Sepahacel after tryptic digestion [10].

2.3. LC20 exchange

An 8 to 10 molar excess of LC20 phosphorylated with [γ-^{32}P]ATP (P-LC20) was added to the sample; intact myosin (0.5 mg/ml), 0.5 M NaCl, 30 mM Tris-HCl, pH7.5, in the presence of 1 mM EDTA or 1 μM Mg^{2+} (Ca^{2+}-EDTA buffer) and incubated at 40°C for 30 min, with or without monoclonal antibodies. To investigate the role of the S-1/S-2 junction, HMM or S-1 was substituted for intact myosin. Samples were

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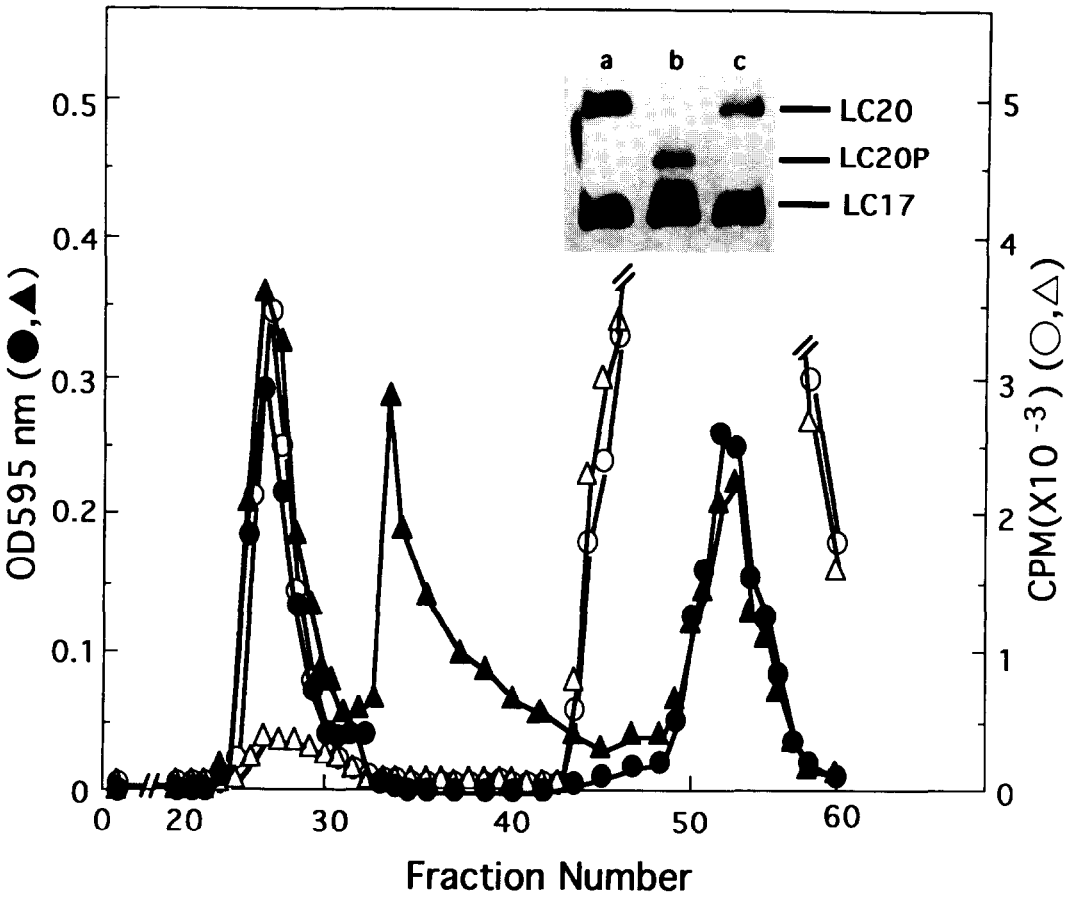

Fig. 1. Elution profile with a SW3000 HPLC column. Radioactivity (○, △) or protein concentration (●, ▲) was shown. LC20 exchange was performed with (△, ▲) or without (○, ●) monoclonal antibody (10 μg/ml). Fraction 25, 34 and 53–54 indicates intact myosin, MM2 antibody or phosphorylated LC20, respectively. Inset shows urea gel-electrophoresis; control myosin (lane a) or myosin fraction exchanged by phosphorylated LC20 in the absence (lane b) or presence (lane c) of MM2 antibody. Specific activity was about 100,000 cpm/1 nmol ATP.

then applied to a SW3000 HPLC column (Toso Co., Japan). Radioactivities in the fractions of myosins, HMM, S-1 or LC20 were counted and protein concentrations of their fractions were measured. The extent of exchange was calculated according to the following formula:

$$\text{Extent of exchange (\%)} = (n + 1)/n \times A/B \times C/2D \times 100$$

where n = exogenous phosphorylated LC20/endogenous LC20, A = amount of the exogenous LC20 in myosin or myosin fragments, B = amount of myosin or myosin fragments, C = molecular weight of myosin or myosin fragments, and D = molecular weight of LC20.

2.4. Others

Urea gel-electrophoresis was performed by the method of Perrie and Perry [14]. Protein concentration was measured by the method of Bradford [15].

3. Results and discussion

The interaction of LC20 with myosin was monitored by measuring the LC20 exchange efficiency. To measure the extent of LC20 exchange, the isolated LC20 was first phosphorylated by MLCK and incubated with myosin at high temperature to accomplish LC20 exchange [7] (also see section 2). Myosin exchanged with exogenous radiolabeled LC20 eluted faster than unbound LC20; efficiency of LC20 exchange was determined according to the radioactivity of LC20 incorporated in myosin. As shown in Fig. 1, the radioactivity of LC20 coeluted with the myosin fraction; urea gel-electrophoresis of the fraction revealed that the LC20 of myosin was almost completely exchanged with exogenous phosphorylated LC20 (Fig. 1, inset lane b). The extent of exchange (%) (see section 2) was calculated as 95–100% in the presence of 1 mM EDTA and about 70% in the presence of 1 μM $MgCl_2$. 1 mM $MgCl_2$ almost completely inhibited the LC20 exchange. On the other hand, when LC20 exchange was performed in the presence of MM2, which recognizes LC17 [10], the incorporation of the exogenous LC20 indicated by radioactivity was markedly diminished (Fig. 1). The decrease in exchangeability was also confirmed by urea gel-electrophoretic analysis which showed a marked decrease in the exogenous phosphorylated LC20 incorporated into myosin (Fig.1, inset lane c). Using this method, we examined the inhibition of LC20 exchange by monoclonal antibodies which recognize various regions of the myosin molecule, i.e. MM2 and MM10 which recognize LC17 [10], MM9 which recognizes S-2 near the S-1/S-2 junction [16], and MM6 which recognizes the head portion of myosin [10]. Table 1 shows the % inhibition of LC20 exchange by monoclonal antibodies in the presence of 1 mM EDTA or 1 μM $MgCl_2$. MM2 inhibited the LC20 exchange most dramatically while MM6, which recognizes the 68-kDa N-terminal fragment of S-1, the top of myosin

Table 1
Percent inhibition of LC20 exchange[a]

Condition of exchange	MM-2			MM-10			MM-9			MM-6
	Intact	HMM	S-1	Intact	HMM	S-1	Intact	HMM	S-1	Intact
1 mM EDTA + 1 mM ATP	85*	85	24	52	ND	ND	29	ND	ND	3
	(5)**	(4)	(3)	(4)			(3)			(2)
1 μM Mg^{2+} + 1 mM ATP	92	90	8	68	35	5	27	21	3	0
	(4)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(2)

ND, not done; intact, intact myosin; HMM, heavy meromyosin; S-1, subfragment-1.
[a]{extent of exchange(− McAb) − extent of exchange(+ McAb)} / extent of exchange(− McAb). Extent of exchange was determined as described in section 2.
*Value shows the mean of 2–5 experiments.
**Values indicate the number of experiments.

S-1 head according to the three-dimensional structure of S-1 [3], did not inhibit the exchange at all. MM10, which also recognizes LC17, inhibited the LC20 exchange but to a lesser extent. This is probably due to the difference in the epitopes of the two antibodies. MM9 inhibited the exchange but the inhibition was significantly less potent than LC17 recognizing antibodies.

Table 2 shows the effects of various antibodies on the actin-activated ATPase activity of dephosphorylated myosin [10,16]. Antibodies except MM6 activated the ATPase activity and the extent of activation was MM2 > MM10 > MM9. The point of interest is that the inhibition efficiency of the antibodies against LC20 exchange is closely correlated to the extent of activation of ATPase activity of myosin. Another important point is that none of the antibodies inhibited the LC20 exchange of S-1 while the inhibition of LC20 exchange for HMM was found to be virtually the same as intact myosin (Table 1). Corresponding to this observation, it was found that all of the antibodies failed to activate acto-S1 ATPase activity (not shown).

Recently it was found that deletion of the C-terminal residues of LC20 decreases the affinity of LC20 for heavy chain [17], and the region critical for the binding to heavy chain was assigned to be Lys^{149}–Ala^{166} [17]. This suggests that the major anchoring site of LC20 to myosin via the LC20–myosin heavy chain interface localizes to the C-terminal domain of LC20. Therefore it is likely that the antibodies strengthened the interaction between LC20 and the heavy chain so as to reduce the exchangeability. The most dramatic inhibition of LC20 exchange was observed with anti-LC17 antibodies and this is of our interest.

Three-dimensional structure analysis of the skeletal muscle S-1 [3] and scallop myosin regulatory domain [4] revealed that the C-terminal domain of regulatory light chain is in close proximity to the N-terminal domain of essential (alkali) light chain. Furthermore, it was revealed for scallop myosin that Gly^{117} in the C-terminal domain of the regulatory light chain interacts with the N-terminal residues of essential light chain [4]. Therefore, it is plausible that the binding of anti-LC17 antibodies to LC17 changes the interaction between the N-terminal domain of LC17 and the C-terminal domain of LC20. An important finding is that such a change in the interaction is reflected by an activation of actomyosin ATPase activity. This view is supported by a recent finding that the elimination of LC17 significantly diminishes actomyosin motility [18]. Quite recently, we found that the residue Lys^{149}–Phe^{158} of LC20 at the C-terminal domain are critical for the phosphorylation-induced activation of actomyosin ATPase activity [17]. Together with this information, it can be explained that anti-LC17 antibodies activate the ATPase activity by altering the interaction between the C-terminal domain of LC20 and the N-terminal domain of LC17, which in turn changes the LC20–heavy chain interaction which is critical for the activation of actin-activated ATPase activity of smooth muscle myosin. The present results suggest that the interaction between LC17 and LC20 is involved in the activation mechanism of smooth muscle myosin molecule.

Table 2
The effect of McAb on the actin-activated ATPase activity of dephosphorylated myosin

McAb	Epitope	V_{max} (nmol/min · mg)	K_{actin} (mg/ml)
–	–	1.85	0.89
MM 2	LC17	23.85*	0.63*
MM 10	LC17	9.62	0.55
MM 9	S-2	5.92**	0.45**
MM 6	S-1	2.02	0.85
P-Myosin	–	25.56*	0.73*

McAb, monoclonal antibody; P-Myosin, phosphorylated myosin; LC17, 17,000 Da myosin light chain; S-1, subfragment-1; S-2, subfragment-2.
*Higashihara et al. [10].
**Higashihara et al. [16].

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