BINDING OF PARATROPOMYOSIN TO MYOFIBRILLAR PROTEIN FROM CHICKEN SKELETAL MUSCLE

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Background

Paratropomyosin, which was isolated for the first time by Takahashi *et al.* (1985), inhibits actin-myosin interactions and induces weakening of their linkages during postmortem ageing of muscle. Such weakening of rigor linkages allows lengthening of the rigor-shortened sarcomeres (Yamanoue and Takahashi, 1988; Takahashi *et al.*, 1995) and accounts for the increased tenderness of meat during ageing. Paratropomyosin is found at the junction of A- and I-bands of sarcomeres in living muscle (Hattori and Takahashi, 1988). The increase of Ca^{2+} concentration to 2×10^{-4} M in postmortem muscle (Takahashi *et al.*, 1992) results in the translocation of paratropomyosin from its original position to thin filaments (Hattori and Takahashi, 1988; Takahashi *et al.*, 1995), where paratropomyosin may affect the interaction of actin and myosin. However, it has not been known what protein or other substance interacts with paratropomyosin at the A-I junction, or why paratropomyosin is released from its original position by the increased calcium concentrations.

Objectives

In this study, we showed that biotinylated paratropomyosin specifically bound to such species of myofibrillar protein as β-connectin and actin on the membrane, in examining binding of paratropomyosin at the A-I junction of sarcomeres, and showed that adding of paratropomyosin to β-connectin solution resulted in the increase of turbidity of the mixture.

Methods

Chemicals — Streptavidin conjugated with alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and 4-nitroblue tetrazolium chloride (NBT) were purchased from Boehringer (Mannheim, Germany). Sulfo-N-hydroxysuccinimide biotin (sulfo-NHS-biotin) was bought from Pierce Chemical Co. (Rockford, Illinois). Other chemicals were of analytical reagent grade.

Proteins — Chicken breast muscle was used. Myofibrils were prepared by the method of Perry and Grey (1956). Paratropomyosin was purified with a hydroxyapatite column as described previously (Yamanoue *et al.*, 1998). β-Connectin was prepared by the method of Kimura and Maruyama (1983) and purified by the method of Itoh *et al.* (1986). Separation of 400 kDa fragment produced by β-connectin digestion by α-chymotrypsin was done by the method of Kawamura *et al.* (1995), with omission of adding of 0.1% SDS. Myosin, actin, tropomyosin, and troponin were prepared according to the commonly used methods.

Assays — Paratropomyosin was biotinylated by incubation with sulfo-NHS-biotin (2:1, molar ratio to paratropomyosin) by the method of Kincaid *et al.* (1988). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done by either the procedure of Laemmli (1970) with 12% and 15% polyacrylamide gels or that of Tatsumi and Hattori (1995) with a 2% polyacrylamide gel containing 0.5% agarose. After SDS-PAGE, the separated proteins were electrotransferred to a nitrocellulose membrane by the method of Towbin *et al.* (1979). The membrane was incubated with biotinylated paratropomyosin for 1 h, and the protein binding to biotinylated paratropomyosin was detected by streptavidin conjugated with alkaline phosphatase and biotin (Kincaid *et al.*, 1988). Turbidity was measured with a spectrophotometer at 320 nm after incubation of protein mixtures at 25℃ for 30 min.

Results and discussions

Biotinylation of paratropomyosin was completed successfully and biotinylated paratropomyosin was specifically detected with streptavidin-biotin on a nitrocellulose membrane (data not shown). Freshly prepared chicken myofibrils were electrophoresed on 12% gel, and transferred onto the membrane. After the transfer myofibrillar protein were stained with naphthol blue black (Fig. 1A). Such proteins as myosin heavy chain, α-actinin, actin, tropomyosin (paratropomyosin), troponin, and myosin light chains were stained. When incubated with streptavidin conjugated with alkaline phosphatase and then with the colorogenic substrates BCIP and NBT for alkaline phosphatase, two polypeptide bands of 80 kDa and 124 kDa appeared (Fig. 1B, lane a). The appearance of these bands was due to non-specific binding of streptavidin to myofibrillar protein. Therefore, the biotinylated paratropomyosin specifically bound to actin, troponin-T and tropomyosin (paratropomyosin) (Fig. 1B, lane b).

This result was confirmed by incubation of each protein prepared from chicken muscle with biotinylated paratropomyosin (Fig. 2B). The bands of actin, paratropomyosin, tropomyosin, troponin-T, and troponin-I, were colored during incubation with BCIP and NBT, but both bands of myosin heavy chain and light chains were not so. It has been shown that paratropomyosin bound to actin, but not to myosin, *in vitro* (Nakamura and Takahashi, 1985). The band of troponin-I was not colored in the result of Fig. 1B, probably because the amount of troponin-I transferred from myofibrils was much less than from troponin molecule. Troponin-C was not transferred onto a nitrocellulose membrane under these conditions.

It is supposed that paratropomyosin may bind to connectin at the A-I junction, because Maruyama *et al.* (1977) have found that connectin is the most abundant compound in the A-I junction of myofibrils, and because Hattori and Takahashi (1988) used indirect immunofluorescence to show that paratropomyosin is to be found only at the A-I junction in fresh myofibrils. To examine this

possibility, we purified β-connectin from myofibrils and evaluated its interaction with paratropomyosin (Fig. 3). After SDS-PAGE using a 2% polyacrylamide gel containing 0.5% agarose, β-connectin was electrotransferred onto the membrane and overlaid with biotinylated paratropomyosin. When the biotinylated paratropomyosin was detected with streptavidin-biotin, the band of β-connectin was colored by the incubation with BCIP and NBT (Fig. 3, lane c). The band of 400 kDa fragment mixed very slightly in a β-connectin solution was also colored. These results showed that paratropomyosin bound to β-connectin on the membrane.

It was not known whether native paratropomyosin bound to β-connectin under undenatured conditions or not, so we measured the changes in turbidity of a β-connectin solution containing 0.1 M NaCl, 1 mM EGTA, and 50 mM MOPS, pH 7.0, caused by adding various amounts of unmodified paratropomyosin (Fig. 4). Turbidity rose until the paratropomyosin added was in a 5:1 weight ratio to 50 μg/ml β-connectin (about 300:1 molar ratio) tested. When paratropomyosin was added to a solution of 400 kDa fragment, turbidity of the mixture was also increased with increased of paratropomyosin. Addition of various amounts of bovine serum albumin to β-connectin solution had no effect on turbidity.

Conclusions

Biotinylated paratropomyosin bound to actin, tropomyosin, paratropomyosin, troponin-T, troponin-I, and β-connectin on a nitrocellulose membrane. Both turbidities of β-connectin and 400 kDa fragment solutions increased with increase of added paratropomyosin. Because both of connectin and paratropomyosin have been found at the A-I junction region in fresh myofibrils, paratropomyosin may interact with connectin filaments at the A-I junction of sarcomeres in living and pre-rigor skeletal muscles.

Literature

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Data in the form of figures

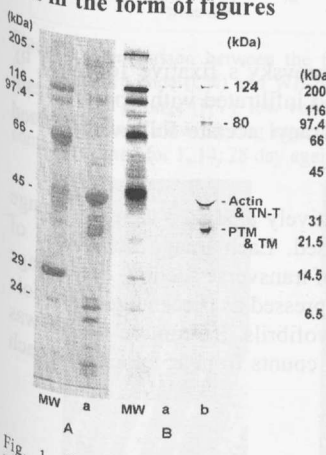

Fig. 1. Detection of Biotinylated Paratropomyosin Bound to Myofibrillar Protein. The membrane was stained with naphthol blue black (A) and detected by streptavidin and biotin (B). Lane a, myofibrils; lane b, myofibrils that had been incubated with biotinylated paratropomyosin; MW, molecular weight marker.

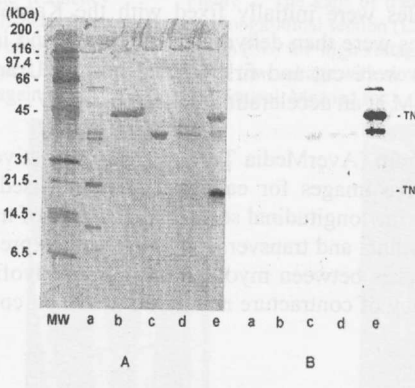

Fig. 2. Binding of Biotinylated Paratropomyosin to Purified Myofibrillar Proteins. Each protein was electrophoresed on a 15% gel. The membrane was stained with naphthol blue black (A) and detected by streptavidin and biotin (B). Lane a, myosin; lane b, actin; lane c, paratropomyosin; lane d, tropomyosin; lane e, troponin; MW, molecular weight marker.

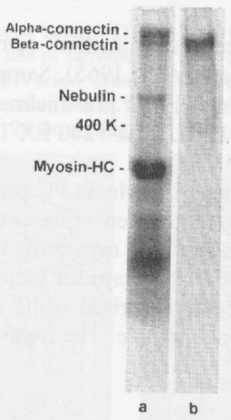

Fig. 3. Binding of Biotinylated Paratropomyosin to β-Connectin. Myofibrils and β-connectin (a and b) were stained with Coomassie brilliant blue R-250 and β-connectin binding to biotinylated paratropomyosin (c) was detected by streptavidin and biotin.

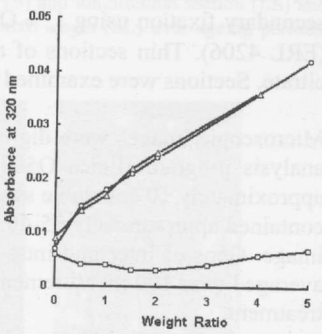

Fig. 4. Effects of Paratropomyosin on the Turbidities of β-Connectin and 400 kDa Fragment Solutions. Various amounts of paratropomyosin were added to β-connectin (○) and 400 kDa fragment (△) solutions. Bovine serum albumin (□) was also added to β-connectin solution.