Interaction of α-actinin and nebulin in vitro

Support for the existence of a fourth filament system in skeletal muscle

Rüdiger Nave, Dieter O. Fürst and Klaus Weber

Max Planck Institute for Biophysical Chemistry, Department of Biochemistry, D-3400 Goettingen, FRG

Nebulin is a high molecular weight polypeptide (mass 0.6–0.8 million) which accounts for 3% of the myofibrillar mass in skeletal muscle. Due to its resistance to extraction under native conditions, relatively little is known about the biochemistry of the molecule. Here we report in vitro binding of α-actinin (a major Z-line protein) to nebulin. After solubilization with sodium dodecylsulfate myofibrillar polypeptides separated by gel electrophoresis were blotted on nitrocellulose and probed with ^{125}I-labelled α-actinin. Nebulin is the only polypeptide decorated by α-actinin. This result gives biochemical support for the hypothesis, based on recent immunoelectron micrographs, that nebulin could form in skeletal muscle a fourth filament system, possibly extending to the Z-line.

α-Actinin; Cardiac muscle; Nebulin; Sarcomere; Skeletal muscle; Z-line

1. INTRODUCTION

In their search for an elastic filament system Wang et al. [1,2] found that approximately 10% of the mass of a skeletal muscle myofibril arises from three unusually large polypeptides: titin TI, titin TII and nebulin. Titin TII is a soluble proteolytic derivative of the larger but non extractable titin TI, while nebulin is a different non extractable protein. A bank of more than 14 distinct monoclonal antibodies recognizing in majority single epitopes was used to trace the course of the titin molecule within the sarcomere by immunoelectron microscopy [3–6] and to relate some of the epitope positions with antibody decoration patterns obtained on the purified titin TII molecule [7]. The results show that the very long titin string, acting as a polar unit, spans the half sarcomere. It is attached via its TI specific physical end to the Z-band [3] and penetrates with the other end deep into the M line [4]. The proteolytic cleavage site giving rise to TII seems located at the N_1 line [7].

Titin is present in all vertebrate sarcomeric muscles. As a third filament organization it seems to integrate the thin filaments of the I/Z/I brushes with the thick filaments (A-bands) during myofibrillogenesis [8]. In contrast attempts to understand the structure and func-

tion of the nebulin molecule have been frustrated by its resistance to extraction under native conditions [1–3]. In addition it seems to play a role unique to skeletal muscle, since various attempts in different laboratories to detect a counterpart in cardiac muscle by immunological and biochemical methods were unsuccessful [3,9–11]. Several immuno-fluorescence and -electron microscopy studies located nebulin essentially to the N_2 line [2,3]. However, some recent immunoelectron micrographs suggest that nebulin could form a fourth filament system, possibly extending close to the Z line [11,12].

In an attempt to understand the linkage of titin and/or nebulin to the Z band by an independent approach, we have used α-actinin, the major F-actin associated protein of the Z band [13] (for a recent review see [14]), in gel blotting studies. Here we report that skeletal muscle myofibrils solubilized by SDS contain in gel blotting experiments a single polypeptide reacting with ^{125}I-labelled α-actinin. This polypeptide, identified as nebulin, is present in skeletal muscle but not in cardiac or smooth muscle. This in vitro result provides the first independent support for the idea [11] that a nebulin-based fourth filament system, present only in skeletal muscle, could extend to the Z-line.

2. MATERIALS AND METHODS

2.1. *Purification and iodination of α-actinin*

The protein purified from chicken breast muscle [15] was subjected to gel filtration on Superose 12 FPLC (HR 10/30, Pharmacia) in phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 1 mM NaN_3, pH 7.3). About 100 μg of the central portion of the protein peak (volume 250 μl) were labelled

Correspondence address: K. Weber, Max Planck Institute for Biophysical Chemistry, Postfach 2841, Am Fassberg, D-3400 Goettingen, FRG

Abbreviations: 2ME, 2-mercaptoethanol; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; BSA, bovine serum albumin

with ^{125}I ($Na^{125}I$, #IMS.30, Amersham) using one bead of iodination reagent (Iodo bead, Pierce, Rockford, IL, USA) as specified by the manufacturer. After 15 min at room temperature the mixture was passed through a PD-10 column equilibrated in PBS containing 3% bovine serum albumin (BSA) to remove free iodine. Labelling specificity was monitored by gel electrophoresis and autoradiography.

2.2. Preparation of crude nebulin

A homogenate of 0.5 g of chicken breast muscle in 5 ml of 50 mM Tris-HCl, pH 7.8, 2 mM 2ME and 4 M urea was centrifuged (1000 × g for 10 min). The resulting pellet was washed twice with the same solvent and the final residue, well enriched in nebulin, was dissolved in 1 ml 8 M urea.

2.3. Gel electrophoresis and binding assay

For optimal gel analysis of the high molecular weight polypeptides in the muscle extracts, extracts were incubated at 50°C for 15 min in the SDS sample buffer rather than boiled [3,7].

Gel electrophoresis on linear polyacrylamide gradient gels (2–12% acrylamide, 0.5% bisacrylamide) and immunoblotting were as described [3]. Monoclonal antibodies specific for nebulin (clone Nb2) and titin (clone T23) were characterized previously [3]. Total muscle tissue solubilized by SDS was subjected to SDS PAGE. Polypeptides were transferred electrophoretically from the gel onto nitrocellulose as done for immunoblotting. Transfer quality was monitored with Ponceau red. Blots were cut into strips and treated with BPM buffer (3% BSA in PBS containing 2 mM 2ME) for 2 h. Individual strips were incubated with iodinated α-actinin (100000 cpm in 1 ml BPM buffer) for 2 h at room temperature. After extensive washing with BPM buffer (at least 5 changes in 24 h) the blots were air dried and autoradiographed on Fuji RX 100.

3. RESULTS

α-Actinin purified from chicken breast muscle was subjected to gel filtration to eliminate aggregated material. An aliquot from the central portion of the peak was labelled with ^{125}I to a specific activity of $0.6–1.2 \times 10^6$ cpm/μg of α-actinin. Fig. 1 demonstrates the purity of the sample used for iodination and the specific incorporation of the label (lane c). The iodinated α-actinin was used in a binding assay on myofibrillar polypeptides separated by SDS PAGE and subsequently transferred to nitrocellulose. The resulting blots were incubated with iodinated α-actinin, thoroughly washed and subjected to autoradiography.

Fig. 2 (lane c) shows that radiolabelled skeletal muscle α-actinin decorates a single polypeptide present on the nitrocellulose blot. From its apparent molecular weight of around 0.6 to 0.8 millions, the polypeptide is recognized as nebulin and this is confirmed by a nebulin specific antibody, which recognizes the same polypeptide (Fig. 2, lane d). When myofibrils are first extracted with 4 M urea, nebulin remains insoluble. The subsequent 8 M urea extract is highly enriched in nebulin (Fig. 2, lanes f and h), which again is strongly decorated by the α-actinin probe (Fig. 2, lane g). In additional experiments in which unlabelled α-actinin was added to the incubation mixture the resulting autoradiographs showed strong reduction of the nebulin labelling.
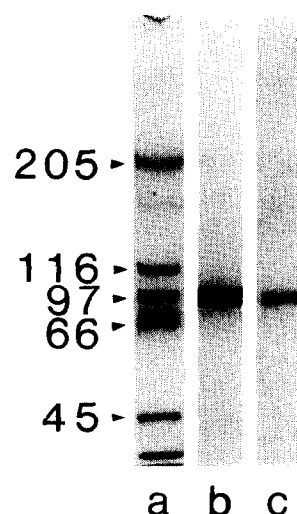

Fig. 1. Iodination of α-actinin purified from chicken breast muscle. Lane b shows the purified α-actinin after the final gel filtration step. The 10% polyacrylamide gel is stained with Coomassie blue. Lane c is the autoradiograph of ^{125}I-labelled α-actinin. Molecular weight standards are indicated in lane a.

Since previous gel analyses and immunological studies did not detect a nebulin related polypeptide in cardiac and smooth muscle [3,9–11] we probed blots of
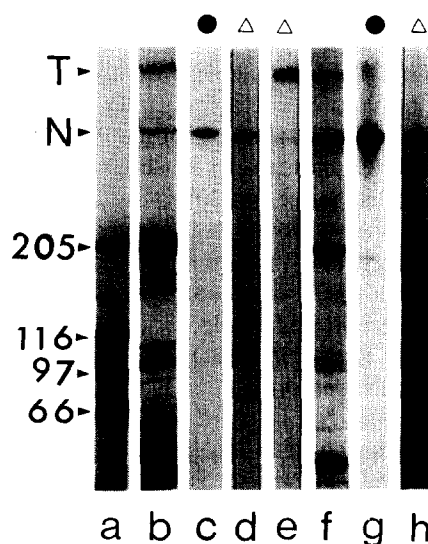

Fig. 2. Binding of radiolabelled α-actinin to nebulin. Samples of skeletal muscle tissue (chicken breast) separated by SDS PAGE were transferred to nitrocellulose. Lane b shows a Ponceau red stained blot. The binding assay monitored by autoradiography reveals strong decoration of a 500000 molecular weight polypeptide (c). Corresponding blots treated with monoclonal antibodies specific for titin (T23; e) and nebulin (Nb2; d) identify the 500 kDa band as nebulin. Lane f shows the Ponceau red stain of a crude fraction highly enriched in nebulin. The corresponding autoradiograph after incubation with ^{125}I-labelled α-actinin shows strong decoration of the nebulin band (g). The same band is recognized by the nebulin antibody (Nb2; h). Molecular weight standards and the positions of titin (T) and nebulin (N) are indicated in lane a. Autoradiographs are marked by circles and immunoblots by triangles.
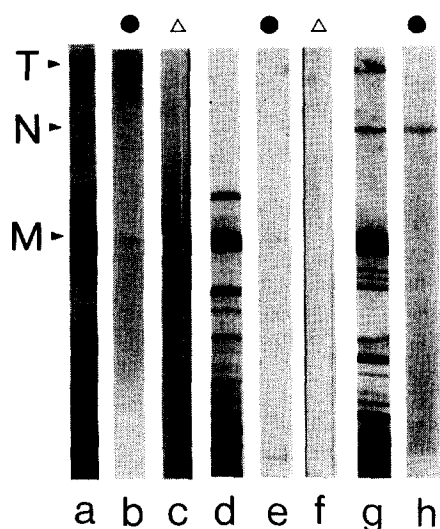
Fig. 3. ^{125}I labelled α-actinin assays on heart and smooth muscle. Ponceau red stained blots of chicken heart and gizzard are shown in lanes a and d, respectively. In both cases the assay with ^{125}I-labelled α-actinin from skeletal muscle did not detect a binding protein (b, e). The monoclonal nebulin antibody Nb2 shows the absence of a corresponding antigen (c, f). The binding assay with iodinated α-actinin from gizzard provided on skeletal muscle tissue (for Ponceau red stain see g) a specific decoration of the nebulin band (h). The positions of titin (T), nebulin (N) and myosin heavy chain (M) are indicated. Autoradiographs are marked by circles and immunoblots by triangles.

chicken heart and chicken gizzard with the iodinated α-actinin. Fig. 3 (lanes a–f) shows that no polypeptide was recognized by the α-actinin probe. Several experiments were also made with α-actinin purified from gizzard. The labelled smooth muscle α-actinin reacted on nebulin in skeletal muscle (Fig. 3, lane g,h) but did not detect a binding protein in blots of smooth and cardiac muscle.

4. DISCUSSION

We have shown that radiolabelled α-actinin used on nitrocellulose blots of myofibrillar polypeptides previously separated by SDS PAGE decorates exclusively the nebulin polypeptide in the case of skeletal muscle tissue. The reaction is specific as it is retained by polypeptide fractions highly enriched in nebulin and can be competed with unlabelled α-actinin. In addition, in line with the absence of nebulin from cardiac and smooth muscle [3,9–11] the α-actinin probes do not detect a corresponding target in these muscle tissues. Since nebulin was thought for a long time to be restricted to the N_2 line [2,3] an in vitro complex formation of α-actinin and nebulin would not have been understood. However, recent immunoelectron micrographs obtained with a polyclonal antibody detected additional nebulin epitopes along the half sarcomere [11,12]. One of them is located about 100 nm from the Z-band. These results led Wang and Wright

to the hypothesis of a set of parallel inextensible nebulin filaments possibly anchored at the Z-band of skeletal muscle sarcomeres [11]. Our finding of a complex between α-actinin and nebulin provides the first independent support for the nebulin filament model and yields a previously unexpected molecular property of α-actinin.

Although the ultrastructure of the Z-band of skeletal muscle has been extensively analyzed by electron microscopy [16], relatively little is known about its constituents except for α-actinin and F-actin (for minor putative Z-line constituents see [17–20]). As an antiparallelly oriented dimer the α-actinin molecule can crosslink neighbouring F-actin filaments [21]. In smooth muscle α-actinin sequence analysis defines an EF hand Ca^{2+} binding site preceding the actin binding site, which is followed by several spectrin-like repeats. These repeats are responsible for the rod-like structure of the molecule. In α-actinin from sarcomeric muscle the Ca^{2+} sites are non-functional due to changes in the EF hand (reviewed in [14]). Our results indicate that at least in skeletal muscle the functions of α-actinin are more complex than previously thought. Since the domain structure of α-actinin is well established, proteolytic derivatives or recombinant fusion proteins can now be used to locate the nebulin binding site on α-actinin. While the absence of nebulin filaments from cardiac muscle remains a puzzle [3,9–11], it may be related to the known length heterogeneity of actin filaments in the same I segment [11].

As in any overlay assay, the experiments with α-actinin require successful renaturation of the binding site of the corresponding ligand. While this seems easily achieved with nebulin, the negative results on other muscles do not exclude the presence of putative α-actinin binding proteins which escape detection by our assay. Similarly, our results still leave open how titin filaments are anchored at the Z-band.

REFERENCES

[1] Wang, K., McClure, J. and Tu, A. (1979) Proc. Natl. Acad. Sci. USA 76, 3698–3702.

[2] Wang, K. and Williamson, C.L. (1980) Proc. Natl. Acad. Sci. USA 77, 3254–3258.

[3] Fürst, D.O., Osborn, M., Nave, R. and Weber, K. (1988) J. Cell. Biol. 106, 1563–1572.

[4] Fürst, D.O., Nave, R., Osborn, M. and Weber, K. (1989) J. Cell Sci. 94, 119–125.

[5] Whiting, A., Wardale, J. and Trinick, J. (1989) J. Mol. Biol. 205, 263–268.

[6] Maruyama, K., Yoshioka, T., Higuchi, H., Ohashi, K., Kimura, S. and Natori, R. (1985) J. Cell Biol. 101, 2167–2172.

[7] Nave, R., Fürst, D.O. and Weber, K. (1989) J. Cell Biol. 109, 2177–2188.

[8] Fürst, D.O., Osborn, M. and Weber, K. (1989) J. Cell Biol. 109, 517–527.

[9] Hu, D.H., Kimura, S. and Maruyama, K. (1986) J. Biochem. 99, 1485–1492.

[10] Locker, R.H. and Wild, D.J.C. (1986) J. Biochem. 99, 1473–1484.

[11] Wang, K. and Wright, J. (1988) J. Cell Biol. 107, 2199–2212.

[12] Maruyama, K., Matsuno, A., Higuchi, H., Shimaoka, S., Kimura, S. and Shimizu, T. (1989) J. Muscle Res. Motil. 10, 350–359.

[13] Robson, R.M. and Zeeche, M.G. (1973) Biochim. Biophys. Acta 295, 208–224.

[14] Blanchard, A., Ohanian, V. and Critchley, D. (1989) J. Muscle Res. Motil. 10, 280–289.

[15] Bretscher, A., Vandekerckhove, J. and Weber, K. (1979) Eur. J. Biochem. 100, 237–243.

[16] Yamaguchi, M., Iumimoto, M., Robson, R.M. and Stromer, M.H. (1985) J. Mol. Biol. 184, 621–644.

[17] Casella, J.F., Casella, S.J., Hollands, J.A., Caldwell, J.E. and Cooper, J.A. (1989) Proc. Natl. Acad. Sci. USA 86, 5800–5804.

[18] Maher, P.A., Cox, G.F. and Singer, J.F. (1985) J. Cell Biol. 101, 1871–1883.

[19] Ohashi, K. and Maruyama, K. (1989) J. Biochem. 106, 110–114.

[20] Suzuki, A., Saito, M., Okitani, A. and Nonami, Y. (1981) Agric. Biol. Chem. 45, 2535–2542.

[21] Wallraff, E., Schleicher, M., Modersitzki, M., Rieger, D., Isenberg, G. and Gerisch, G. (1986) EMBO J. 5, 61–67.