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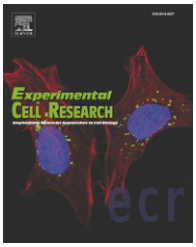

Research Article

Evidence for γ-actin as a Z disc component in skeletal myofibers

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ABSTRACT

We investigated the targeting of the γ-actin isoform in skeletal myofibers. For this purpose we used expression vectors to produce green fluorescent protein (GFP-) as well as myc-tagged γ-actin in rat *flexor digitorum brevis* myofibers. We found that the γ-actin fusion proteins accumulated into Z discs but not beneath the sarcolemma. Instead, the GFP-tagged skeletal muscle-specific α-actin isoform was preferentially incorporated into the pointed ends of thin contractile filaments. The localization pattern of the γ-actin fusion proteins was completely different from that of the dystrophin glycoprotein complex on the sarcolemma. The results emphasize the role of γ-actin as a Z disc component but fail to reveal an actin-based sub-sarcolemmal cytoskeleton in skeletal muscle cells.

Introduction

The sarcolemmal membrane of adult skeletal myofibers is an organized structure that is thought to form a link to the contractile apparatus. The dystrophin–dystroglycan complex has been shown to function as a trans-membrane linker between the extracellular matrix and the cytoskeleton [1,2]. The sarcolemmal α7β1 integrin is likely to have a similar role [3]. However, the identity of the cytoskeletal molecules mediating the interaction further to the contractile apparatus has remained disputable. Accordingly, it is not exactly known what protein or proteins mediate the lateral force transmission from the contractile apparatus to dystrophin and possibly to integrin. Neither is it clear what molecules are responsible for the cross-striated organization seen on the myofiber surface. One strong candidate is the cytoplasmic actin because it has been reported to be present beneath the sarcolemma. Accordingly, Lubit and Schwartz [4] used an anti-actin antibody that did not recognize α-actin and suggested that it marked the sarcolemma. Similarly, Craig and Pardo [5] reported that γ-actin, spectrin, intermediate filament proteins, and vinculin were organized into sub-sarcolemmal belt-like structures that were called costameres. A more recent study using γ-actin specific monoclonal antibodies arrived at a similar conclusion [6]. It has also been reported that the costameric γ-actin colocalizes with dystrophin [7]. Furthermore, β-spectrin, vinculin, and talin that interact with actin have been found beneath the sarcolemma of skeletal myofibers [8,9] and *in vitro* studies have shown interaction between dystrophin and γ-actin [10]. Moreover, in yeast two-hybrid analysis has shown direct interaction between F-actin and β-dystroglycan [11].

It is notable, however, that there is no unanimity concerning the localization of cytoplasmic actin in skeletal myofibers, which is not surprising owing to the huge excess of the contractile α-actin and the close similarity between the actin isoforms. Accordingly, Otey et al. [12] who produced muscle and non-muscle actin-specific antibodies did not observe any marked difference between

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the distribution patterns of the sarcomeric α-actin and the cytoplasmic γ-actin. Nakata et al. [13] raised anti-peptide antibodies against a γ-actin specific amino acid sequence and found γ-actin exclusively in the Z discs. Kee et al. [14] also used γ-actin specific antibodies and drew the conclusion that γ-actin is located in Z discs and regions adjacent to them. Thus the immunolocalization studies of γ-actin have been inconclusive.

Here we have analysed the distribution of tagged γ-actin in rat skeletal muscle cells. In this study tagged γ- or α-actin were produced in isolated myofibers with two different methods. We either infected isolated myofibers with a recombinant Semliki Forest virus [15], or transfected muscle cells in live animals by electroporation [16]. Our results indicate that both GFP-γ-actin and myc-γ-actin located to the Z discs. GFP-α-actin, on the contrary, marked the pointed ends of thin filaments. We did not see subsarcolemmal localization for GFP-γ-actin in the isolated myofibers or in the muscle sections obtained from electroporation-transfected animals. One could assume that there is an interaction between the Z disc-associated GFP-γ-actin and the sarcolemmal dystrophin glycoprotein complex at the Z line circumference where the two proteins could make contact but we failed to demonstrate colocalization between these components in our confocal microscopy studies. The results support the view of γ-actin as a Z disc component but they do not support the idea of a sub-sarcolemmal actin-based cytoskeleton.

Methods

Cell cultures

Myofibers were isolated from the footpad of female Spraque–Dawley rats. Briefly, rats were killed and the *flexor digitorum brevis* (FDB) muscles were excised and digested with collagenase as described [17]. The single fibers obtained were plated on Matrigel-coated (Beckton-Dickinson Labware) dishes and cultivated for 16 h in MEM (Gibco, UK) containing 5% horse serum before experiments. L6 rat myoblasts were obtained from ATCC and grown for 2 days in DMEM (Gibco, UK) containing 7% fetal calf serum.

Plasmids, recombinant viruses, infections and electroporations

A plasmid encoding GFP-human β-actin (pEGFP-actin) was from Clontech (CA) and pSFV-GFP-β-actin was kindly provided by Prof. Kalervo Väänänen (University of Turku, Finland). pSFV has been described by [15]. The amino acid sequence of rat γ-actin (NP_001120921) and human β-actin are identical except for four amino acids in the amino terminus. Accordingly we changed the cDNA encoding GFP-human β-actin in pEGFP-actin and pSFV-β-actin plasmids by *in vitro* mutagenesis so that it encodes the amino acid sequence of rat γ-actin. The mutagenesis was performed with QuickChange Site-Directed Mutagenesis Kit (Stratagene GmbH, Germany). Sequencing using an ABI PRISM™ 3100 sequencer and BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA) was used to verify that desired nucleotide sequence was obtained. Cloning to pSFV1 was performed as described [15]. Myc tag was cloned to pSFV1 and sequence coding the γ-actin amino acid sequence was inserted to this modified vector. Recombinant SFV particles encoding GFP-γ-actin or myc-γ-actin were prepared in BHK-21 cells as described by [18]. Typically the virus stocks exhibited multiplicities of 10^7–10^8 as analysed on BHK cell monolayers. To infect muscle cells virus stock was placed on the isolated myofibers in appropriate dilution and the virus was allowed to adsorb for 3–8 h. After adsorption the virus was removed and the infection was allowed to proceed in culture media as indicated. L6 cells were infected similarly but shorter time periods.

The α-actin cDNA sequence (NM_019212) was generated by PCR from oligo(dT)-primed rat muscle cDNA. The amplification was performed using *Taq* DNA polymerase (Amplitaq gold, Perkin Elmer; 94 °C, 10 min 94 °C, 40 s; 54 °C, 40 s; 72 °C, 2 min; 30 cycles; 72 °C, 7 min). The PCR-product was cloned to the pAcGFP1-C1 vector (Clontech, CA). The subcloned fragment was sequenced using an ABI PRISM™ 3100 sequencer and BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA).

GFP-α-actin, GFP-γ-actin or plain-GFP plasmid was introduced into rat FDB muscle by means of electroporation as described earlier [16]. The plasmid DNA (1, 2–3, 2 μg/μl PBS) was injected into the muscle through a small skin incision in the hind paw and a train of 10 pulses 20 ms each at 200 V/cm aimed at the paw was generated using a ECM 830 pulse generator (BTX Genetronics Inc.). After 3–7 days from the operation the animal was sacrificed and the FDB excised, and either digested with collagenase to obtain single myofibers, or the muscle was fixed by immersion in 3% paraformaldehyde in PBS for 2 h and then frozen with isopentane cooled with liquid nitrogen for cryo-sectioning (8 μm).

All the animal experiments were approved by the State Provincial Office of Finland and the Laboratory Animal Centre of the University of Oulu.

Fluorescence studies

Mouse monoclonal anti-β-dystroglycan and anti-dystrophin antibodies were obtained from Novocastra Laboratories (Newcastle, UK). Monoclonal anti-α-actinin and anti-desmin antibody and TRITC-phalloidin were obtained from Sigma Chemical Co. Monoclonal anti c-myc antibody was obtained from Santa Cruz Biotechnology Inc., CA. Monoclonal anti-paxillin antibody was from BD Transduction Laboratories. Alexa 568- and Alexa 488-conjugated anti-mouse IgG were obtained from Molecular Probes (Leiden, Netherlands). For immuno-fluorescence confocal microscopy, the immuno-labeled cultures were examined with a ZEISS LSM 510 Laser Scanning microscope equipped with Argon (excitation 488 nm) and HeNe (excitation 543 nm) lasers (Carl Zeiss Inc, Germany). Intensity profiles were produced by using the LSM510 Pascal software (Carl Zeiss, Germany). Images were processed with Adobe Photoshop®6.

FRAP measurements

FRAP measurements were performed on myofibers transfected with plasmids by means of electroporation. Confocal microscope LSM 510 equipped with 100× objective and argon laser (488 laser line) was used to bleach outlined region of interest (ROI). Temperature was kept at 23±1 °C in CO_2-independent medium containing glutamine, penicillin-streptomycin and 5% horse serum. GFP-actins were bleached with minimum laser power and 70–100% transmittance for 13–25 s. The fluorescence recovery was followed for 15 min with 15–30 s scanning intervals at minimum laser power and 8% transmittance. Because of its rapid