An historical perspective of the discovery of titin filaments –Part 2

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Received: 15 December 2017 / Accepted: 18 December 2017 / Published online: 13 January 2018

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Abstract

In 2017, a Special Issue of *Biophysical Reviews* was devoted to “Titin and Its Binding Partners. The issue contained a review: “An historical perspective of the discovery of titin filaments” by dos Remedios and Gilmour that was intended to be a history of the discovery of the giant protein titin, previously named connectin. The review took readers back to the earliest discovery of the so-called third filament component of skeletal and cardiac muscle sarcomeres and ended in 1969. Recently, my colleague Shin’ichi Ishiwata gently reminded me of two papers published in 1990 and 1993 that were unwittingly omitted from the original historical perspective. In the first paper (J Cell Biol 110:53–62, 1990), Funatsu et al. examined the elastic filaments in skeletal muscle using a combination of light and electron microscopy, but they also measured resting as well as passive stiffness mechanical measurements to establish that connectin (titin) is responsible for both stiffness and fiber tension. In the second paper (J Cell Biol 120:711–724, 1993), Funatsu et al. used permeabilised cardiac muscle myocytes (from rabbit papillary muscles) and focussed on filament ultrastructure using either freeze-substitution or deep-etched replica methods to visualise connectin/titin filaments in fibers with and without actin and myosin filaments.

Keywords Connectin filaments · Titin filaments · Skeletal muscle myocytes · Cardiomyocytes · Ultrastructure · Sarcomeric protein

Funatsu et al. (1990) used optical microscopy and conventional electron microscopy of glycerinated and mechanically skinned single rabbit psoas muscle fibres that were freed of extracellular collagen. Thin filaments were dissolved using gelsolin and their removal confirmed by the absence of staining with fluorescent-phalloidin (fluorescence microscopy) or decoration with myosin subfragment-1 (electron microscopy). Gelsolin is superior to DNase I and formamide, both of which adversely affect the Z discs. They also monitored passive elastic tension and stiffness before and after removal of the thin filaments. The high sensitivity of connectin to proteolysis by dilute trypsin enabled them to selectively remove connectin to reveal the presence of another giant protein, nebulin (~ 780 MDa, not found in cardiac muscle). Nebulin was located in the I band between the Z disc and the so-called N line located about 0.1 μm away from the edge of the A band. The authors concluded that nebulin probably does not contribute to the passive elasticity and, therefore, that connectin is the principal

elastic element in the sarcomere. The attachment of connectin to each end of the myosin thick filaments enabled the authors to confirm the conclusion of Horowitz et al. (1986), namely that connectin is therefore responsible for restoring the A band to the middle of the sarcomere.

In the second paper, Funatsu et al. (1993) produced a technically superb investigation of the filamentous ultrastructure of rabbit papillary muscle connectin (titin). The authors included Koscak Maruyama, the discoverer of connectin. The reason this protein eluded earlier detection is because it was literally the “elephant-in-the-room.” Connectin/titin at nearly 4 MDa is nearly 20-fold larger than the heavy chain of the muscle motor protein myosin (200 kDa). Biophysicists were clearly not accustomed to the idea of such an unwieldy protein. Connectin and other giant proteins (see below) tend to be both insoluble and “sticky”, i.e. they have multiple binding partners. Furthermore, a single connectin molecule is large enough to span half the sarcomere, with its N terminus buried in the Z discs and its C-terminus located at the central M line where it binds to a connectin molecule from the other half of the sarcomere.

The authors introduced several novel approaches that threw light on the filamentous structure and function of connectin

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filaments. They also used gelsolin to efficiently depolymerise actin filaments, thus facilitating the distinction between actin thin filaments and connectin thin filaments, and removed 80% of the myosin filaments using 1M buffered potassium acetate under relaxing conditions (Mg-ATP, no free Ca). Frozen tissue sections of gelsolin-treated sarcomeres produced remarkably clear images of connectin filaments spanning the sarcomeric I bands. This method produced excellent ultrastructure images of rabbit papillary muscle, although the resolution was insufficient to visualise fine details on the connectin/titin filaments. However, the authors improved the quality of myofilament imaging using the freeze substitution of deep-etch replica technique, producing some remarkably detailed images of the connectin filaments, including an ~ 4 nm axial repeat. The preparation of deep-etched replica of the sarcomeres was not simple. Briefly, a sample of rabbit papillary muscle was chemically skinned, cryosectioned at -150 °C, evacuated under nitrogen at high vacuum (2×10^6 torr), warmed to -100 °C, deep-etched and then rotary shadowed with platinum and carbon at different shadow angles. The tissue was then immersed in methanol and dissolved in bleach, following which

Fig. 1 **a** Image of a freeze-substituted longitudinal section rabbit papillary muscle from which both the actin-containing thin filaments and the myosin-containing thick filaments have been selectively removed using gelsolin and high salt, respectively. Note the two M lines (M) marking the middle of their A bands, and the single Z disc (Z) connected to the M lines by poorly resolved connectin/titin filaments. **b** A stereo pair of the equivalent region of the sarcomere using a deep-etch replica method. The projections along the connectin filaments exhibit an ~ 4 nm repeat (reproduced with permission from the Rockefeller University Press, *Journal of Cell Biology* 1993: p. 722, Fig. 12)

the Pt-C replicas were floated onto water, transferred to formvar-coated grids and photographed as stereo pairs by electron microscopy (Fig. 1).

Panel a of Fig. 1 shows a thin section of freeze-substituted cardiac muscle treated to extract both thin filaments (gelsolin) and thick filaments (1M acetate). The original M lines (M) and a Z disc (“Z”) are labelled, revealing thin filaments that were difficult to resolve. The two images of panel b of Fig. 1 are a stereo pair of the same extracted filaments observed using the deep-etched technique. Connectin filaments exhibit a 4-nm repeat that probably corresponds to the sequential 100-residue immunoglobulin connectin domains. The connection between the connectin and myosin filaments led the authors to speculate that connectin might determine the length of the thick filaments. In the same paper, the authors “decorated” the thin filaments with myosin subfragment-1, which showed that while some thin filaments exhibited the classical “arrowhead” motif, others (connectin) were undecorated.

Finally, in keeping with the dos Remedios-Gilmour (2017) review, the authors constructed a model showing the likely arrangement of actin and myosin filaments with connectin filaments weakly/randomly associated with actin filaments (Fig. 2a) and how the connectin filaments appear when actin is removed.

What next?

More recently, we have drawn attention to the possible role of yet another giant but smaller (900 kDa) protein called obscurin (Marston et al. 2015). Obscurin is arranged in a reverse sense to connectin, meaning its N terminus is at the M

Fig. 2 A model of the three-dimensional network of connectin/titin filaments. **a** The thick (myosin-containing), thin (actin-containing) and connectin (elastic) filaments are shown. Connectin filaments join the Z disc to the ends of the thick filaments and make weak lateral connections to the parallel thin filaments. **b** Actin-containing thin filaments have been removed, and the connections to the thin filaments at the junction line are lost (reproduced with permission from the Rockefeller University Press, *Journal of Cell Biology* 1993: p. 723 Fig. 13)

line, and its C-terminus, although still to be precisely defined, extends towards the Z disc. Obscurin has been implicated in familial human dilated cardiomyopathy, but this has yet to be proven (Vikhorev et al. 2017). The N-terminus of obscurin binds to the C-terminus of connectin/titin (Qadota et al. 2017), but it can also bind to the small isoform of titin called Novex-3 located near the Z disc (Kellermeyer et al. 2017). Clearly there is more work to be done with these giant proteins before we can claim to understand how sarcomeres really function.

Acknowledgements The author is grateful for the financial assistance of Medical Advances Without Animals for the purchase of a nitrogen vapour tissue freezer. He is also grateful to Dr Shin'ichi Ishiwata for his comments on the manuscript.

Compliance with ethical standards

Conflict of interest Cristobal G. dos Remedios declares that he has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by the author.

References

dos Remedios C, Gilmour D (2017) An historical perspective if the discovery of titin filaments. *Biophys Rev* 9:179–188

- Funatsu T, Higuchi H, Ishiwata S (1990) Elastic filaments in skeletal muscle revealed by selective removal of thin filaments with plasma gelsolin. *J Cell Biol* 110:53–62
- Funatsu T, Kono E, Higuchi H, Kimura S, Ishiwata S, Yoshioka T, Maruyama K, Tsukita S (1993) Elastic filaments in situ in cardiac muscle: deep etch replica analysis in combination with selective removal of actin and myosin filaments. *J Cell Biol* 120:711–724
- Horowitz R, Kempner ES, Bisher ME, Podolsky RJ (1986) A physiological role for titin and nebulin in skeletal muscle. *Nature (London)* 323:160–164
- Kellermeyer D, Smith JE 3rd, Granzier H (2017) Novex-3, the tiny titin of muscle. *Biophys Rev* 9:201–206. <https://doi.org/10.1007/s12551-017-0261-y>
- Marston S, Montgiraud C, Munster AB, Copeland O, Onjee C, dos Remedios C, Messer AE, Ehler E, Knöll R (2015) OBSCN mutations associated with dilated cardiomyopathy and haploinsufficiency. *PLoS One* 10:e0138568. <https://doi.org/10.1371/journal.pone.0138568>
- Qadota H, Matsunaga Y, Nguyen KCQ, Mattheyses A, Hall DH, Benian GM (2017) High-resolution imaging of muscle attachment structures in *Caenorhabditis elegans*. *Cytoskeleton* 74:426–442. <https://doi.org/10.1002/cm.21410>
- Vikhorev PG, Smolktunowicz N, Munster AB, Copeland O, Kostin S, Montgiraud C, Messer AE, Toliat MR, Li A, dos Remedios CG, Lal S, Blair CA, Campbell KS, Guilin M, Knoll R, Marston SB (2017) Abnormal contractility in human heart myofibrils from patients with dilated cardiomyopathy due to mutations in TTN and contractile protein genes. *Sci Rep* 7:1–11. <https://doi.org/10.1038/541598-017-13675-8>