Independent Assembly of 1.6 μm Long Bipolar MHC Filaments and I-Z-I Bodies

H. Holtzer¹, T. Hijikata², Z.X. Lin³, Z.Q. Zhang³, S. Holtzer¹, F. Protasi¹, C. Franzini-Armstrong¹, and H.L. Sweeney⁴

¹Dept. of Cell and Developmental Biology, Univ. of Penn., Philadelphia, PA 19104, ²Dept. of Anatomy, Medical School, Univ. of Gunma, Maebashi, Japan, ³Dept. of Cell Biology, Beijing Institute for Cancer Research, Beijing, China, and ⁴Dept. of Physiology, Medical School Univ. of Penn., Philadelphia, PA 19104

The proposition that the penultimate step in the assembly of striated myofibrils (SMFs) requires the interlocking of two pre-assembled intermediate structures derives from several disparate observations. The first is related to the assembly of thick filaments independently of both thin filaments and Z-bands. Antin *et al.* (1) and Toyama *et al.* (31) demonstrated that in Taxol-treated postmitotic, mononucleated myoblasts or multinucleated myotubes (a) nascent 1.6 μm long bipolar thick filaments interdigitated, not with thin filaments, but with long microtubules and (b) in such regions Z-bands were also wanting. In Taxol-treated cells clusters of thick filaments often aligned laterally, forming A bands with pseudo-H zones, M-bands, etc. The second observation is related to the assembly of I-Z-I bodies, precursors to the definitive I-Z-I brushes. Schultheiss *et al.*, Lu *et al.* and Lin *et al.* (18, 19, 27) reported that irregularly-shaped s- α -actinin/titin T20/ α -actin aggregates, 0.2–0.8 μm in size, assembled and aligned longitudinally in the absence of thick filaments. These assemblages were observed in regions of myogenic cells just hours prior to the emergence of definitive SMFs. Microscopically such assemblages resembled stress fibers in non-muscle cells and so were termed “stress fiber-like structures”. Stress fiber-like structures differ from their counterparts in non-muscle cells in that they consist of sarcomeric α -actinin, α -actin, troponin, tropomyosin, titin and nebulin, but lack sarcomeric myosin and their associated proteins. In contrast, authentic stress fibers in non-muscle cells consist of non-sarcomeric α -actinin, β - or γ -actin, caldesmon, non-sarcomeric myosin, etc. Authentic stress fibers are rare in myogenic cells that have up-regulated the synthesis of myofibrillar isoforms (16, 17). In developing muscle stress fiber-like structures rapidly intermix with preformed 1.6 μm MHC filaments. Such structures, positive for both 1.6 μm bipolar MHC filaments and I-Z-I bodies have been termed “non-striated myofibrils” (11, 12).

Together these observations led us to propose that in normal development, it was the 3-dimensional intermingling of longitudinally-oriented, pre-assembled MHC-positive thick filaments with pre-assembled I-Z-I bodies that accounted for the images of non-striated myofibrils in both the light and electron microscopes. The emergence of definitive A bands and 0.1–0.2 μm wide Z-bands within the confines of the non-striated myofibrils depended upon two events: (1) the translocation and sorting-out of the preformed 1.6 μm long bipolar MHC filaments and the I-Z-I bodies, and (2) the reconfiguration of the Z-band proteins into the square lattice arrangement characteristic of mature I-Z-I brushes (25). Fixing the length of the α -actin/tropomyosin/troponin thin filaments was a late event in assembling tandem sarcomeres; it occurred only after the formation of definitive A- and Z-bands, and is the last step in the transition of I-Z-I bodies into I-Z-I brushes.

In this report we provide additional evidence for this model. First we describe observations on BC3H cells, a line of rhabdomyosarcoma cells (20, 26). Under conditions permitting differentiation, BC3H cells assemble large numbers of randomly-oriented “free-floating A-bands”. The assembly of these scattered A bands occurs in the absence of morphologically-recognizable thin filaments and Z-bands. In the presence of Taxol, and again independently of thin filaments and Z-bands, nascent scattered A bands in BC3H cells align longitudinally to form long “pseudo-striated” myofibrils. Secondly, we describe some events associated with the *de novo* assembly of 1.6 μm long bipolar thick filaments and with the nucleation of the I-Z-I bodies. The salient findings of these experiments are that: (1) If 1.6 μm bipolar MHC filaments self-assemble *in vivo* then only a brief period (minutes?) elapses before myomesin and/or titin complex with these nascent MHC polymers; and (2) The initiation of the assembly of I-Z-I bodies requires the concurrent, cooperative and stoichiometric interactions of s- α -actinin, the N-terminal of titin, the C-terminal of nebulin and the s- α -actinin binding site of α -actin.

Author for correspondence: Dr. Howard Holtzer, Dept. of Cell and Developmental Biology, Medical School, University of Pennsylvania, Philadelphia, PA 19104.

Tel: 215–898–8089; Fax: 215–898–9871

RESULTS

I. Assembly of "free-floating A-bands" in BC3H cells in the absence of thin filaments and Z-bands:

The notion that "mini-sarcomeres" are intermediate structures that gradually transform into mature sarcomeres originally stems from observations made on developing muscles in invertebrates (3, 4, 7). In lower forms short thick filaments interdigitating with thin filaments assemble between closely-spaced Z-bands. With further maturation, the thick filaments progressively lengthen, resulting in increasing distance between Z-bands. Sanger and coworkers (21, 22, 23) have interpreted some of their observations on cultured chick myogenic cells along similar lines.

In developing vertebrate myogenic cells, however, the concept of mini-sarcomere is difficult to reconcile with 3 observations: (1) the continuous addition of fully formed $\sim 2.0\ \mu\text{m}$ sarcomeres at the distal tips of elongating myofibrils (11, 12, 19, 27), (2) the assembly of morphologically normal A-bands in Taxol-treated postmitotic, mononucleated myoblasts and myotubes (1, 31); and (3) the emergence of free-floating A-bands in normal cultured cardiomyocytes and postmitotic myoblasts independently of nearby thin filaments or Z-bands (18, 19, 27). These considerations prompted us to screen atypical myogenic cells for comparable free-floating A-bands. Such A-bands have now been observed in cells cloned from 3 cell lines — BC3H, L6 and a Ni-induced rhabdomyosarcoma.

BC3H cells reared in growth medium replicate but do not assemble morphologically-recognizable myofibrillar structures. When reared in mitogen free medium BC3H cells are induced to up-regulate the muscle-regulatory factor myogenin, but they do not up-regulate MyoD (24, 30, 33). As differentiating cells they assemble numerous randomly-oriented free-floating A-bands (Fig. 1). These normal appearing, scattered A-bands assemble in the absence of recognizable thin filaments and Z-bands.

Staining BC3H cells grown under a variety of conditions with combinations of labeled antibodies confirms and extends the EM observations. Figs. 2A and 2C show two different differentiated mouse BC3H myoblasts, double-stained with antibodies to MHC and Rho-phalloidin (Fig. 2B) and anti-desmin (Fig. 2D) respectively. To interpret these images of differentiated mouse BC3H myoblasts more readily, Figs. 2E and 2F show a double-stained mouse C2C12 multinucleated myotube with a normal complement of SMFs (Fig. 2E reveals the localization of anti-MHC, Fig. 2F of Rho-phalloidin). The similarity between the randomly-oriented $1.6\ \mu\text{m}$ long bipolar MHC filaments in the BC3H myoblasts with the more oriented $1.6\ \mu\text{m}$ bipolar MHC filaments (arrows in Fig. 2E) and/or A bands in the C2C12

cells is obvious. Note too that the fine $1.6\ \mu\text{m}$ bipolar MHC filaments in the 2 phenotypes aggregate laterally to merge imperceptibly into typical $1.6\ \mu\text{m}$ wide A bands. The % of BC3H myoblasts with MHC-positive filaments varied from roughly 5% to over 40% in different cultures. The cause of this variation is unknown. However, it is to be emphasized that the anti-MHC did not localize diffusely throughout the cytoplasm and in no instance did it stain a structure that might be interpreted as a mini-sarcomere. If there were MHC filaments less than $1.6\ \mu\text{m}$ long, they escaped our detection. In diameter, however they varied from just perceptible to over $1.0\ \mu\text{m}$ (see Discussion). With the exception of the very finest, most $1.6\ \mu\text{m}$ bipolar filaments displayed a fluorescence-free center of the same dimension as that observed in the H-zone of the normal A bands in either chick (not shown) or mouse SMFs (e.g. Fig. 2E). Antibodies to platelet MHC (10) or to brain MHC (5, 6, 21) did not stain the $1.6\ \mu\text{m}$ bipolar MHC filaments. We found no evidence to suggest that in the assembly of these free-floating A-bands there is a still earlier stage in which non-sarcomeric MHC is replaced by sarcomeric MHC. It is of interest to speculate as to what the finest $1.6\ \mu\text{m}$ bipolar MHC structure might represent. Given the sensitivity of the fluorescence microscope, they may in fact be images of single native thick filaments.

The emergence of $1.6\ \mu\text{m}$ wide A-bands and $0.1\text{--}0.2\ \mu\text{m}$ wide Z-bands in chick SMFs is correlated temporally and spatially with the emergence of myomesin and titin T20 positive stripes. The myomesin forms a stripe in the center of the nascent A-bands, whereas titin T20, together with s-a-actinin precisely costain nascent, narrow Z-bands (18, 19, 27). With these considerations in mind we asked whether in differentiating BC3H myoblasts there was a correlation between the apparent diameters of the $1.6\ \mu\text{m}$ bipolar MHC filaments and their binding of antibodies to either myomesin or titin T20. We found that the finest MHC filaments appeared to be negative for both, whereas the great majority of "thicker" MHC filaments were positive (Figs. 3A–3F). When bound, the anti-myomesin was localized as a fleck at the middle of the MHC filament; it did not stain any structure in the cytoplasm. In contrast the relationships between the MHC filaments and titin were more ambiguous. Anti-titin T20 always localized as a fleck toward the ends of the thicker MHC filaments (see below), whereas most frequently it was absent at the ends of the finer MHC filaments. However, it was also localized as flecks throughout the cytoplasm with no relationship to the $1.6\ \mu\text{m}$ MHC filaments. Whether this failure to detect the association of myomesin and/or titin with the very fine MHC filaments marks an optical limit of our microscopic technique, or reflects a temporal sequence in the assembly of native thick filaments requires further analysis.

Fig. 1. EM sections illustrating the random-orientation of numerous free-floating A-bands. In size, orderly-lateral packing, as well as presence of M bands and pseudo-H zones, the A-bands in Fig. 1A are remarkably similar to those in normal myogenic cells despite the absence of both thin filaments and Z-bands. Long arrows point to M-bands in longitudinal sections, short arrows to thick filaments in cross-sections. Fig. 1B documents the presence of numerous "bridges," possibly at the M-band level, connecting neighboring thick filaments. Bar in Fig. 1A = 0.5 μm ; in Fig. 1B = 0.2 μm .

In normal muscle cells non-striated myofibrils invariably pre-date and pre-figure the emergence of definitive SMFs. By definition, non-striated myofibrils consist of disordered, over-lapping arrays of 1.6 μm long thick filaments and I-Z-I structures in various stages of maturation. Given the absence of all I-Z-I bodies, it is not surprising that in differentiating BC3H cells no structure approaching non-striated myofibrils was ever observed.

BC3H cells are tumor cells. Conceivably the bipolar MHC filaments and/or the A-bands that assemble in such cells have different properties from their counterparts in normal myogenic cells. For example, the super-numerary microtubules induced by Taxol in normal chick myogenic cells might serve as surrogate thin filaments for nascent thick filaments, resulting in long

"pseudo-striated" myofibrils (1, 31). Interestingly, despite lacking thin filaments and Z-bands, the Taxol-induced pseudo-striated myofibrils in chick myoblasts stained for titin T20, T12, and T30. Would BC3H myoblasts reared in Taxol respond as did normal chick myoblasts? As illustrated in Figs. 4A and 4B the pseudo-striated myofibrils induced by Taxol in BC3H cells are indistinguishable from the similar structures induced in similarly treated normal chick myogenic cells.

Consistent with the cytological data, *s*- α -actinin was not detected in SDS/polyacrylamide gels or Western blots of differentiated BC3H cells. Unexpectedly, however, while not present in replicating cells, in differentiating BC3H myoblasts some form of α -actin did accumulate. The fate of this α -actin isoform is being investi-

Fig. 2. Double-stained fluorescence micrographs of differentiating mouse BC3H myoblasts (Figs. 2A–2D) and mouse C2C12 myotubes (Figs. 2E and 2F) illustrating the virtual identity of (a) nascent $1.6\ \mu\text{m}$ long bipolar MHC filaments and (2) $1.6\ \mu\text{m}$ wide A bands in the two phenotypes. The BC3H myoblasts and the C2C12 myotubes were double-stained with anti-MHC (Figs. 2A, 2C, and 2E) and either Rho-phalloidin (Figs. 2B and 2F) or anti-desmin (Fig. 2D). Note in Figs. 2A, 2C and 2E the fluorescent-free zone at the center of all the bipolar MHC structures. Comparable fluorescent-free zones — corresponding to the bare zone observed in EM — are not obvious in the very fine MHC filaments that are well below the resolution of the fluorescence microscope. The longitudinal orientation of the single A bands marked by arrows in Fig. 2E probably reflect the unstained longitudinally oriented stress fiber-like structures present in such regions (for details see 1, 18, 27).

Fig. 3. Double-stained BC3H myoblasts reared in differentiation medium. Figs. 3A, 3C and 3E reveal the localization of anti-MHC, whereas Fig. 3B is stained with Rho-phalloidin, and Figs. 3D and 3F with anti-myomesin. There is no detectable relationship between the distribution of Rho-phalloidin and of the bipolar MHC filaments or A-bands. The center of most bipolar MHC structures bind the anti-myomesin (Figs. 3C and 3D). Nevertheless there is an ill-defined cut-off such that 1.6 μ m MHC filaments below a still to be determined diameter do not display a recognizable myomesin center (Figs. 3E and 3F).

Fig. 4. A differentiated BC3H myoblast reared in Taxol. Fig. 4A reveals the localization of anti-MHC, Fig. 4B that of titin T20. Compare the more longitudinally aligned pseudo-striated myofibrils in these cells with those illustrated in Figs. 1, 2 and 3. Essentially similar images have been observed in Taxol-treated cells costained with MHC and titin T12 and T30.

gated. Currently, we are attempting to induce Z-bands by transfecting these cells with full length and mutated *s*- α -actinin cDNAs (10, 28).

In summary: (1) Not only will native MHC fibrils assemble in BC3H cells, but in such cells they align laterally, forming remarkably normal but randomly-oriented A bands. Given the limit of resolution of the fluorescence microscope, the precise relationship, both temporal and spatial, of titin and myomesin to nascent MHC bipolar filaments requires further study. Nevertheless it is highly likely that as soon as a "few" native thick filaments aggregate laterally, the complex is positive for both myomesin and titin.

II. Probing the properties of mature I-Z-I brushes in myotubes transfected with s- α -actin/MYC cDNAs:

In mature I-Z-I brushes the highly ordered *s*- α -actinin/titin T20/ α -actin definitive Z-bands display a regular $\sim 2.0 \mu\text{m}$ periodicity. In EM sections each Z-band measures $0.1\text{--}0.2 \mu\text{m}$ in width and is characterized by a crystalline-like square lattice. In contrast, the precursor electron-opaque Z-bands in I-Z-I bodies display an irregular $\sim 0.8 \mu\text{m}$ periodicity (Sanger *et al.*, 1986). More-

over in I-Z-I bodies the variably-shaped *s*- α -actinin/titin T20/ α -actin precursor Z-band masses measures $0.2\text{--}0.8 \mu\text{m}$ in size. To follow the progressive transformation of precursor $0.2\text{--}0.8 \mu\text{m}$ *s*- α -actinin Z-bands into the narrow, highly ordered definitive $0.1\text{--}0.2 \mu\text{m}$ wide Z-bands we have constructed four *s*- α -actinin cDNAs each tagged with a MYC-epitope. The four cDNAs are: (1) Full length (FL/MYC), (2) Minus EF-hand Ca^{2+} motifs ($^{-}\text{EF/MYC}$), (3) Actin-binding site only ($^{+}\text{A/MYC}$), and (4) Minus actin-binding site ($^{-}\text{A/MYC}$).

To provide appropriate background, we first describe the distribution of the expressed *s*- α -actinin/MYC peptides in the better known I-Z-I brushes. Schultheiss *et al.* (28) reported that in transfected day 4–5 myotubes both untagged full length and untagged *s*- α -actinin lacking C-terminal EF-hand Ca^{2+} motifs were selectively incorporated into preexisting Z-bands. (Note: The equally important finding that *the continued expression of these peptides in day 8 and older myotubes had distinctly different effects, will not be discussed here. For details see* (10, 28).) To confirm these findings and to demonstrate that the MYC-tag did not perturb normal tar-

getting of nascent exogenous peptides, primary myogenic cultures were transfected with the four peptides. Day 4–5 transfected myotubes, which are rich in terminally differentiated SMFs, were double-stained with (a) antibodies to MYC, and (b) antibodies to endogenous s - α -actinin, α -actinin, titin T20, T12 and T30, as well as with antibodies to nebulin, troponin I, tropomyosin, MHC, MyBP-C, and myomesin.

All four s - α -actinin/MYC peptides were incorporated rapidly, selectively and exclusively into preexisting Z-bands (e.g. Figs. 5A–5D). Cytoplasmic staining was not observed. There was no evidence in these immature myotubes that the expressed peptides by intercepting endogenous “soluble” proteins induced bizarre ectopic myofibrillar aggregates. It is worth noting that the selective incorporation into Z-bands in the I-Z-I brushes applied to the s - α -actinin/MYC peptides lacking actin-binding sites as well as with those with intact actin-binding sites. Finding that all four s - α -actinin/MYC pep-

tides were promptly targeted to the preexisting Z-bands was unexpected, given their very different behavior in PtK2 cells (e.g. Hijikata *et al.*, 1994). These findings are also relevant to the issue of which site(s) on the s - α -actinin molecule are critical for targeting and/or incorporation into the Z-band lattice.

Double-staining with (a) MAb anti-MYC and polyclonal anti- s - α -actinin, or (b) polyclonal anti-MYC and MAb anti-titin T20, revealed that without exception every MYC-positive Z-band was a chimeric structure — i.e. positive for exogenous s - α -actinin/MYC, as well as for endogenous α -actinin and titin T20. In day 4–5 myotubes these chimeric Z-bands were morphologically indistinguishable from those in untransfected myotubes. The formation of chimeric Z-bands had no adverse effects on the assembly of flanking thick or thin filaments, as determined by double-staining with antibodies to MHC, myomesin, MyBP-C, α -actinin, tropomyosin or troponin I. Similarly, antibodies to titin T20,

Fig. 5. Double-stained day 4 and day 7 normal chick myotubes transfected with (a) s - α -actinin/MYC cDNA lacking its actin binding site (Fig. 5A), and (b) s - α -actinin/MYC cDNA with only its actin binding site (Fig. 5C). Both expressed peptides were localized with anti-MYC. Both transfected myotubes were also stained with anti-vinculin (Figs. 5B and 5D). The morphologically normal Z-bands in both were also positive for endogenous s - α -actinin. The selective incorporation into preexisting Z-bands of full length or s - α -actinin/MYC lacking EF-hand Ca^{2+} domains was indistinguishable from those shown here. Note the absence of any obvious relationship between the vinculin-positive specks in Fig. 5B with the overlying Z-bands. The changes in vinculin distribution in the plane of the sarcolemma in the more mature myotube shown in Fig. 5C is accompanied by talin and integrin. Figs. 5B and 5D demonstrate that whatever role “costomeres” might serve in cultured cardiomyocytes (19) their role in the early stages of myofibrillogenesis in skeletal muscle is not obvious.

T12 and T30, as well as to nebulin were localized in their expected positions relative to the MYC-positive Z bands. On the other hand when younger myofibrils with MYC-positive Z-bands with a $\sim 2.0 \mu\text{m}$ periodicity were costained with antibodies to α -actin, tropomyosin or troponin I they stained continuously, giving the impression they were still non-striated. Similarly, despite the presence of both periodic A and Z-bands, *these myofibrils stained continuously when decorated with Rhophalloidin* (e.g. Figs. 2E and 2F). That the fixing of the length of thin filaments at $\sim 1.0 \mu\text{m}$ is a late event in maturing I-Z-I brushes has been noted before (2, 9, 18, 29).

In Summary: (1) The MYC-tag did not interfere with the normal targeting of nascent $s\text{-}\alpha$ -actinin/MYC peptides, nor did it perturb the assembly of definitive I-Z-I brushes; (2) unexpectedly the incorporation of $s\text{-}\alpha$ -actinin/MYC into preexisting Z-bands can occur without the participation of EF-hand Ca^{2+} motifs, spectrin repeats, or even actin-binding domains, and (3) the last

Fig. 6. Double-stained growth up of a day 4 myotube transfected with a full length $s\text{-}\alpha$ -actinin cDNA. Fig. 6A reveals the localization of anti-MYC, Fig. 6B the localization of anti- α -actin. Virtually every detectable irregularly-shaped MYC-positive aggregate is associated with α -actin.

changes in filament length does not involve the further elongation of thick filaments as postulated by the “mini-sarcomere” model, but involves adjusting the length of the thin filaments.

III. Using $s\text{-}\alpha$ -actinin/MYC to follow the nucleation of I-Z-I bodies:

Whether assembled in (a) cultured cardiac myocytes, (b) new-born, postmitotic, mononucleated myoblasts, or (c) multinucleated myotubes, linearly aligned I-Z-I bodies have been defined as irregularly-shaped $s\text{-}\alpha$ -actinin/titin T20/ α -actin aggregates, $0.2\text{--}0.8 \mu\text{m}$ in size (18, 27, 28). Aggregates of this size positive for titin T20 but negative either for $s\text{-}\alpha$ -actinin or α -actin, have not been

Fig. 7. Double-stained day 4 myotube transfected with $s\text{-}\alpha$ -actinin/MYC cDNA lacking EF-hand Ca^{2+} motifs. Fig. 7A reveals the localization of anti-MYC, Fig. 7B the localization of anti-nebulin. Note that in the transfected myotube and in the transfected postmitotic, mononucleated myoblast, each MYC-positive aggregate is also nebulin-positive. There was no suggestion that titin or $s\text{-}\alpha$ -actinin accumulated prior to α -actin or that temporally one of these proteins functioned as a scaffold or nucleation center for the others. Judging from the Chronological Table in Lin *et al.* (18) we estimate that the new-born mononucleated myoblast in this Figure was roughly 15 h. old. Extensions (arrow) from postmitotic myoblasts to myotubes are common in these cultures — presumably they are involved in the still poorly understood event of fusion.

observed. Similarly $s\text{-}\alpha\text{-actinin}$ aggregates have not been localized in the absence of titin T20 or $\alpha\text{-actin}$. In contrast, the association of troponin I, tropomyosin, or titin T12 and T30 with small $s\text{-}\alpha\text{-actinin}$ aggregates is more variable.

The arrangement of I-Z-I bodies into stress fiber-like structures is most conspicuous in regions where SMFs will shortly emerge (2, 5). One particularly favorable location is the growth tips of maturing myotubes. Accordingly, to study the conditions affecting the nucleation of I-Z-I bodies we have focused on the growth tips of chick myotubes transfected with our four $s\text{-}\alpha\text{-actinin}$ /MYC cDNAs. All of the expressed $s\text{-}\alpha\text{-actinin}$ /MYC peptides behave similarly — all four were incorporated selectively into nascent I-Z-I bodies (e.g. Figs. 6 and 7). As noted for the MYC-positive Z-bands in I-Z-I brushes, the precursor Z-bands in the I-Z-I bodies were also chimeric structures staining for both exogenous $s\text{-}\alpha\text{-actinin}$ /MYC and endogenous $s\text{-}\alpha\text{-actinin}$. Furthermore, every MYC positive I-Z-I body precisely costained with antibodies to titin T20 and $\alpha\text{-actin}$. These findings suggest that the cytoplasmic signals that guide

the incorporation of endogenous $s\text{-}\alpha\text{-actinin}$ into nascent I-Z-I bodies are equally efficient in guiding nascent exogenous MYC-tagged molecules.

Prompted by the finding that the C-terminal of nebulin inserts into the Z-band of I-Z-I brushes, we have recently double-stained I-Z-I bodies in (a) untransfected myotubes, using polyclonal anti- $s\text{-}\alpha\text{-actinin}$ and MAb anti-nebulin, and (b) transfected myotubes using polyclonal anti-MYC and MAb anti-nebulin. The I-Z-I bodies in both series invariably costained for $s\text{-}\alpha\text{-actinin}$ and nebulin. In no instance was either endogenous $s\text{-}\alpha\text{-actinin}$ or exogenous $s\text{-}\alpha\text{-actinin}$ /MYC localized to a I-Z-I body that was negative for nebulin (Figs. 7A and 7B). Conversely, in regions rich in stress fiber-like structures, nebulin was not found independently of $s\text{-}\alpha\text{-actinin}$ /MYC or $\alpha\text{-actin}$. The simplest interpretation of these findings is that the nucleation of all I-Z-I bodies requires the concurrent and cooperative interaction of $s\text{-}\alpha\text{-actinin}$, the N-terminal of titin, the C-terminal of nebulin and the $s\text{-}\alpha\text{-actinin}$ binding site of $\alpha\text{-actin}$.

Fig. 8. A model for the assembly of SMFs based on observations made on normal chick skeletal and cardiac myogenic cells, on cultured mouse C2C12 myotubes, and mouse BC3H myoblasts. See Discussion for details.

DISCUSSION

Figure 8 is a cartoon summarizing the observations in this report. This modular model raises 3 basic issues: (1) What is the minimal number of myofibrillar proteins required for the *de novo* assembly of a single 1.6 μm bipolar MHC filament, or a single I-Z-I body?; (2) Do molecules such as titin and nebulin, which have been postulated to serve as "molecular rulers" for thick (13, 15) and thin (32) filaments, respectively, play major roles in the interdigitation of the preformed thick and thin filaments?; and (3) How can one account for both the nucleation and reconfiguration of irregularly-shaped 0.2–0.8 μm precursor Z-bands in I-Z-I bodies into conserved, definitive narrow Z-bands in I-Z-I brushes?

The free-floating A bands in normal and Taxol-treated myoblasts and in BC3H cells, demonstrate that 1.6 μm MHC filaments can assemble and aggregate into A bands independently of I-Z-I structures. Despite caveats regarding the resolution and sensitivity of our immunocytochemical procedures, the data suggest that neither myomesin nor titin is required for the assembly of single 1.6 μm bipolar MHC filaments. The failure to detect myomesin until after bipolar MHC filaments have assembled in BC3H myoblasts is consistent with similar findings in chick myoblasts and cultured cardiomyocytes (18, 19, 27). The failure to detect titin until after MHC filament assembly suggests that titin's role should be reconsidered. Rather than a molecular ruler for the thick filament, we propose that titin acts as a molecular ruler for the entire sarcomere. This is by virtue of the fact that its N-terminus is part of the Z-line, while its extended C-terminal region attaches to preformed thick filaments, in essence "capturing" them (and perhaps bundling and orienting them) into the sarcomere.

The intrinsic capacity of MHC monomers to self-assemble into homopolymers of constant diameter but of different lengths *in vitro* is well known (e.g. Huxley, 1963). Less clear is whether *in vivo* other proteins play critical roles in determining the 1.6 μm length conserved in all vertebrate thick filaments. If our failure to detect titin juxtaposed to nascent fine MHC filaments is not an optical artifact, then the role of titin may require reconsideration. One possibility is that the extended C-terminal region of titin captures the preformed MHC filaments and integrates them into the extended α -actin/nebulin thin filaments issuing from the I-Z-I bodies. If titin functions in this manner during normal myofibrillogenesis, it is also clear that in BC3H myoblasts titin can capture MHC-filaments even when it is not anchored by its N-terminal in I-Z-I structures.

Little is known of the conditions that, (1) regulate the nucleation of individual I-Z-I bodies, (2) align them longitudinally into stress fiber-like structures, or (3) trans-

form them into mature I-Z-I brushes with extended, oppositely polarized $\sim 1.0 \mu\text{m}$ thin filaments. However, the finding that neither titin nor s- α -actinin has been detected in irregularly-shaped 0.2–0.8 μm aggregates (Figs. 6 and 7) without the simultaneous presence of both nebulin and α -actin, is consistent with the notion that the *de novo* assembly of a single I-Z-I body requires, at a minimum, the simultaneous interaction of these 4 proteins. The forced expression in BC3H cells of MYC-tagged proteins that may be involved in the nucleation and maturation of I-Z-I structures holds promise for delineating this basic problem in myofibrillogenesis.

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