Distinct Families of Z-line Targeting Modules in the COOH-terminal Region of Nebulin

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Abstract. To learn how nebulin functions in the assembly and maintenance of I-Z-I bands, MYC- and GFP-tagged nebulin fragments were expressed in primary cultured skeletal myotubes. Their sites of incorporation were visualized by double staining with anti-MYC antibodies to myofibrillar proteins, and FITC- or rhodamine phalloidin. Contrary to expectations based on in vitro binding studies, none of the nebulin fragments expressed in maturing myotubes were incorporated selectively into I-band ~1.0–μm F-α-actin-containing thin filaments. Four of the MYC/COOH-terminal nebulin fragments were incorporated exclusively into periodic ~0.1–μm Z-bands whereas both anti-MYC and Rho-phalloidin stained intra-Z-band F-α-actin oligomers, only the latter stained the pointed ends of the polarized ~1.0–μm thin filaments. Z-band incorporation was independent of the nebulin COOH-terminal Ser or SH3 domains. In vitro cosedimentation studies also demonstrated that nebulin SH3 fragments did not bind to F-α-actin or α-actinin. The remaining six fragments were not incorporated into Z-bands, but were incorporated (a) diffusely throughout the sarcoplasm and into (b) fibrils/patches of varying lengths and widths nested among normal striated myofibrils. Over time, presumably in response to the mediation of muscle-specific homeostatic controls, many of the ectopic MYC-positive structures were resorbed. None of the tagged nebulin fragments behaved as dominant negatives; they neither blocked the assembly nor induced the disassembly of mature striated myofibrils. Moreover, they were not cytotoxic in myotubes, as they were in the fibroblasts and presumptive myoblasts in the same cultures.

Key words: Z-bands • myogenesis • I-Z-I bands • α-actin • phalloidin staining

Introduction

Given the conservation, complexity and densely packed configuration of Z-discs (Morris et al., 1990; Goldstein et al., 1991; Vigoreaux, 1994; Schroeter et al., 1996; Gregorio et al., 1999), it might be expected that the expression of the mutants and/or fragments of I-Z-I proteins, which lacked appropriate binding sites, would not be incorporated into their normal location in maturing striated myofibrils (SMFs), but would behave as dominant negatives, interfering with the assembly of normal sarcomeres and probably be cytotoxic. However, we reported that expression of 14/16 MYC/sarcomeric α-actinin (s-α-actinin) peptides that lacked their actin and/or titin binding sites, as well as those lacking their four spectrin repeats or calmodulin binding sites, was promptly incorporated exclusively into normal precursor and mature Z-discs when expressed in transfected maturing myotubes (Schultheiss et al., 1992; Holtzer et al., 1997; Lin et al., 1998; Ojima et al., 1999). Despite binding/bundling filamentous actin (F-actin) in cell-free systems, MYC/s-α-actinin did not bind to the polarized ~1.0–μm long F-α-actin-containing thin filaments in differentiating myotubes. It was suggested that, in maturing myotubes, the binding between s-α-actinin and F-α-actin along the ~1.0–μm thin filaments might be blocked by tropomyosin and/or troponin complexes, whereas the s-α-actinin binding sites along the actin oligomers within the Z-band (Yamanouchi et al., 1985) remained available for the incorporation of the MYC/s-α-actinin mutants. Unexpectedly, these truncated MYC/s-α-actinin peptides did not assemble into ectopic structures, behave as dominant negatives, nor were they obviously cytotoxic.
Of the 16 mutants, only the MYC/s-α-actinin lacking the EF-hands and titin binding domain induced hypertrophied Z-bands, or nemaline-like bodies. In brief, MYC/s-α-actinin fragments that would have been predicted to be assembly-incompetent on the basis of in vitro binding studies were, in fact, incorporated into normal precursor I-Z-I bodies and mature Z-band structures when expressed in maturing myotubes. Such apparent discrepancies between in vitro and in vivo binding prompted us to determine whether they reflected unique properties of the following: (1) s-α-actinin as a multifunctional ligand; (2) the presence of available and unsaturated receptors in maturing intra-Z-band structures; or (3) cell type-specific homeostatic controls, which enable maturing myotubes to cope with malformed MYC/s-α-actinin molecules and/or structures (e.g., differential turnover, posttranslational changes of improperly configured peptides, etc.). Accordingly, the same experimental protocols used with MYC/s-α-actinin fragments have now been used to follow the temporospatial incorporation of MYC/COOH-terminal nebulin fragments into I-Z-I bands in maturing day 4-10 myotubes.

Nebulin, a giant actin binding protein (≈800 kD), is a component of vertebrate skeletal sarcomeres (Wang and Williamson, 1980; Wang and Wright, 1988; Kruger et al., 1991; Labeit et al., 1991; Wright et al., 1993; Labeit and Kolmerer, 1995). In situ, a single molecule is incorporated into, and is coextensive in length with, ≈1.0-μm polarized I-band thin filaments (Wang and Wright, 1988; Wright et al., 1993). Its N terminus extends to the pointed end of the F-α-actin thin filament complex, whereas its COOH terminus is an intra-Z-disc component. On the sequence level, nebulin consists of ≈185 modules, depending on the respective isoform (Labeit and Kolmerer, 1995). Each module is made up of ≈35 amino acid residues, which contain a central SDXXYS consensus motif. The central 154 modules are organized into 22 super-repeats. Each super-repeat consists of seven modules, which are thought to reflect the periodicity of the ≈1.0-μM F-α-actin thin filament complex. Recombinant nebulin, containing 2-15 modules, as well as single synthetic nebulin modules, display a strong affinity to F-actin in vivo in vitro binding assays, but truncated modules do not (Rott and Wang, 1994; Pfuhl et al., 1994, 1996; Gonsior et al., 1998; Zhang et al., 1998). Nebulin’s COOH terminus appears to be located 25-30 nm inside the Z-line (Mllevoi et al., 1998). This part of nebulin involves COOH-terminal modules M177-M185, plus a Ser-rich region containing multiple phosphorylation sites, and a COOH-terminal SH3 domain. How this COOH-terminal region of ≈400 amino acids might interact with such intra-Z-disc peptides as F-α-actin, s-α-actinin, T-cap, or the NH2-terminal ≈900 amino acids of titin is unclear (Wang et al., 1996; M aruyama, 1997; M ues et al., 1998; Politou et al., 1998; G autel et al., 1999; G regorio et al., 1999). The activity of Ser and SH3 domains in the assembly and disassembly of cytoskeletal structures has invited speculations that they might be involved in the assembly of Z-bands during myofibrillogenesis.

Experiments were designed to determine which nebulin modules, when expressed in transfected skeletal myogenic cells, might do the following: (1) be incorporated exclusively into Z-discs; (2) bind continuously along the polarized ≈1.0-μM F-α-actin-containing thin filaments; (3) assemble into ectopic structures; (4) interfere as dominant negatives with either the assembly or maintenance of normal I-Z-I structures; or (5) be cytotoxic. Moreover, by following the changing distribution of exogenous MYC/nebulin modules over time, we could also determine whether day 10 myotubes could cope with aberrant nebulin peptides more efficiently than day 4 myotubes.

**Materials and Methods**

**Cell Culture and Transfection Procedures**

Primary cultures of myogenic cells were obtained from a day 11 chick embroyonic pectoral muscle (Antin et al., 1986). Trypsinized cells were plated onto collagen-coated 24 well coverslips (Pro-Plastics) at an initial density of 4.5 × 10^4 cells per 35-mm culture dish. 24-h cultures were transfected with various MYC/nebulin or GFP/nebulin constructs, using a calcium phosphate precipitation method (L et al., 1998; Ojima et al., 1999). Transfection reagents were purchased from Ependorf-5Prime, Inc. (80303).

**Constructs Preparations**

**pDNA3-myc Nebulin.** All nebulin constructs were obtained from a human leg muscle cDNA library (CLONTECH Laboratories Inc.). 10-30 ng of total cDNA was amplified by 25 cycles of PCR amplification, using 10 s 95°C for denaturation, and 6 min 68°C for combined primer annealing/fragment extension. Primer sequences were derived from nebulin’s human cDNA sequence (EMBL data library X83957), and located at module boundaries as inferred from sequence alignments of M1-M185 (Pfuhl et al., 1996); M175S, 5'-tttagtgt acca cctga agg atga gatg cca aag cc-3'; M185S, 5'-tttaggtt cca g aagg g aag cca gctagg-3'; SH3R, 5'-tttaggtt cca g aagg g aag cca gctagg-3'; SerR, 5'-tttaggtt cca g aagg g aag cca gctagg-3'; and M183R, 5'-tttaggtt cca g aagg g aag cca gctagg-3'. M160-M92, the 12th super-repeat region of nebulin, contained the fragments of SR12R1S-M183R. M171-M183, COOH-terminal nebulin 24 modules without Ser-rich and SH3 domains, contained the fragments of M160-M183R. M160-M175, just outside of the Z-bands, contained the fragments of M160-M175R. M171-M183, outside and inside area of the Z-band nebulin modules, contained the fragments of M175-M184R. M175-SH3, nebulin COOH-terminal 11 modules and Ser-rich domain plus SH3 domain, contained the PCR products of M175-SH3R. M175-Ser, nebulin COOH-terminal region without SH3 domain, contained the fragments of SR12R1aS-M183R. M160-M183, COOH-terminal nebulin modules without Ser-rich and SH3 domains, contained the fragments of M160-M183R. M171-M183, outside and inside area of the Z-band nebulin modules, contained the fragments of M175-M184R. M175-SH3, nebulin COOH-terminal 11 modules and Ser-rich domain plus SH3 domain, contained the fragments of M175-M184R. M175-Ser, nebulin COOH-terminal region without SH3 domain, contained the fragments of SR12R1aS-M183R. The following constructs were prepared using the PCR technology (Invitrogen; G ojima et al., 1999). **pEGFP-C1-nebulin.** Nebulin M86-M92 fragments, the 12th super-repeat region of nebulin, were amplified with a calcium phosphate precipitation method (Lin et al., 1998; Ojima et al., 1999). Transfecting these constructs induced ectopic structures; (4) interfere as dominant negatives with either the assembly or maintenance of normal I-Z-I structures; or (5) be cytotoxic. Moreover, by following the changing distribution of exogenous MYC/nebulin modules over time, we could also determine whether day 10 myotubes could cope with aberrant nebulin peptides more efficiently than day 4 myotubes.

**Antibodies**

To detect the expressed MYC-tagged peptides, monoclonal anti-myc antibody (body 1:10) or rabbit polyclonal anti-myc antibody (body 1:200; Upstate Biotechnology) was used. Monoclonal anti-myc antibodies were produced from the hybridoma M YC 9E 10. A nAbs specific to various sarcomeric proteins were used. 5-α-actinin was localized with an ab body 1:200; 9A 2B 9 (1:400).
Lin et al., 1998). mAbs against cardiac α-actin (1:10; clone A cl-20-4.2) and against skeletal α-actin (1:400; clone SC5) were purchased from A merican Res earch Products and Sigma Chemical Co., respectively. They do not stain actin isoforms in nonmuscle cells (Lin et al., 1987; Franke et al., 1996). Intra-Z-band titin epitopes were localized with polyclonal antititin Z1-Z2 (1:50; Gregorio et al., 1998). To visualize endogenous nebulin, monocolonal antinebulin (1:50; NB 2) was purchased from Sigma Chemical Co. To visualize intra-Z-band nebulin, anti-SH3 antibody was used (1:10; M il ler et al., 1998). Myosin heavy chains (MHCs) were labeled with an mAb (F59; 1:20; M il ler et al., 1989) and a polyclonal (O rganon Teknika Corp.). A affinity-purified rabbit antitropomyosin (1:400) was purchased from Organon Teknika Corp.; its specificity for l-band filaments was demonstrated in O jima et al. (1999).

Microscopy

Indirect immunofluorescence double staining has been detailed by Lin et al. (1998) and O jima et al. (1999). In brief, day 3–10 cultures were fixed with 2% formaldehyde in PBS for 3 min after washing with PBS. Cultures were permeabilized with 0.5% Triton X-100 in PBS and treated with blocking buffer (BB; 2% BSA in PBS) for 30 min to minimize nonspecific binding. Samples were incubated with primary antibody for 1 h at 37°C. A fter washing with 0.5% Triton X-100 in PBS, specimens were reacted with secondary antibody for 1 h at 37°C. A affinity-purified secondary antibodies (Jackson ImmunoResearch Laboratories) were conjugated with rhodamine, Texas red or FITC. To localize F-actin, some dishes were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Polyscience). Speci mens were mounted in 60% glycerol in PBS containing 2.5% DABCO (1,6-diamidino-2-phenylindole dihydrochloride; Sigma Chemical Co.). Micrographs were re- anal yzed with Adobe Photoshop (version 5.02).

Single- and double-stained cells were also observed using a laser scanning confocal microscope (LSM 510; Carl Zeiss, Inc.), which employed a laser of z-axis of ~2.5 μm optical sections. The staining intensity profiles illustrated in Fig. 4 (B and D) were based on LSM 510 imaging software (version 2.3).

Z-bands, which measure ~0.1 μm in width in EM sections, appear to measure ~0.25 μm in the fluorescence microscope after staining with antibodies to s-α-actinin or antinebulin (Lin et al., 1998; see Fig. 4). Given the limited resolution of the fluorescence microscope and to avoid confusion in the text, the size of fluorescent Z-bands was taken as ~0.1 μm. The slight errors in estimating size resulting from this procedure did not alter our interpretation of the data. Conventional EM sections were prepared and examined in a Type H-800 (Hitachi Ltd.) as described in T oyama et al. (1982) and Lin et al. (1998).

Purification of the Nebulin SH3 Domain

The cDNA coding for the human nebulin SH3 domain (residues 6,610–6,669, EMBL data library No. AC X83957) was cloned into a His-tagged pET vector (Studier et al., 1990). Transformed BL21 (DE3) cells were incubated in 1/4 strength LB (L B) medium containing 50 μg/ml kanamycin. At the end of their logarithmic phase, the cells were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside overnight at 200 rpm at 18°C. The cell pellets were harvested and frozen. The frozen cells were thawed in a lysis buffer (200 mM NaCl, 20 mM Tris-HCl, pH 8.0), 10 mM imidazole, 2 mM β-mercaptoethanol 0.2% (vol/vol), Igepal CA-630 (Sigma Chemical Co.) containing 200 μg/ml lysozyme, and subsequently sonicated. The soluble overexpressed protein was purified by nickel-NTA agarose (QIAGEN) chromatography and eluted with 200 mM imidazole, pH 8.0. The SH3-containing fractions were dialyzed against 20 mM Tris- HCl, pH 8.0, 2 mM EDTA, 1 mM DTT, and further purified by chromatography on a Q-Sepharose resin (Pharmacia LKB). The nebulin SH3 domain eluted at ~250 mM NaCl, and fractions containing the protein were concentrated in a Centriplus concentrator (Amicon) by centrifugation. The purity of the protein was confirmed by SDS-PAGE, and was seen as a protein of ~8 kD.

Sedimentation Assay

In vitro cosedimentation assays were performed as described by van Straaten et al. (1998). The protein concentrations used were in all cases 0.29 mg/ml (38.3 μM) of purified SH3 domain, 0.12 mg/ml (2.8 μM) of F-actin from rabbit skeletal muscle (prepared as described in Bullard et al., 1985) and 0.37 mg/ml (3.7 μM) of α-actinin from rabbit skeletal muscle (prepared by the method of L anger and Pepe, 1980). The proteins were mixed in F-buffer (0.1 M NaCl, 10 mM Tris, pH 7.5, 1 mM MgCl2, 1 mM NaN3) to a total volume of 10 μl. The samples were placed at room temperature for 30 min, and subsequently centrifuged for 30 min in a Beckman airfuge. The pellets were taken up in 30 μl of water, and the supernatants were reduced to a volume of 55 μl in a speedvac. One sixth the volumes of pellets and supernatants were loaded on a 4–20% SDS-PAGE gradient gel (Bio-Rad Laboratories). Proteins were visualized by Coomassie blue staining.

Results

Localization of MYC/M160-M183 and M160-M175

The shafts of >90% of day 4–10 (Fig. 2, A and A’) myotubes are rich in newly assembled mature striated myofibrils (SMFs). Each 1-band consists of ~1.0-μm F-α-actin/tropomyosin/troponin complexes that insert into periodic Z-bands by their barbed ends (Ishikawa et al., 1969). Each ~0.1-μm wide Z-band has been claimed to be positive for s-α-actinin, F-α-actin, titin, nebulin, and T-cap (Lin et al., 1998; Ojima et al., 1999).

Expressed MYC/M160-M183 and MYC/M160-M175 (Fig. 1) were incorporated exclusively into periodic ~0.1-μm filaments in myotubes (Lin et al., 1998). These proteins were resolved by SDS-PAGE, and the purity of the protein was confirmed by Coomassie blue staining (Fig. 1). The purity of the protein was confirmed by Coomassie blue staining.
wide Z-discs in day 4, 6, and 10 myotubes. Double staining with anti-MYC and antibodies to endogenous s-α-actinin (Fig. 3, A and A’) revealed precise costaining of all MYC/I-Z-discs. These MYC/nebulin peptides did not do the following: (1) bind to the ~1.0-μm F-α-actin-containing I-band filaments; (2) accumulate diffusely in the sarcoplasm; (3) assemble ectopic longitudinal fibrils/patches (see below); or (4) behave as dominant negatives by perturbing the morphology of the nascent MYC/I-Z-discs. Double staining with anti-MYC and antibodies to MHC (Fig. 3, C–C’), titin Z1Z2, or tropomyosin (data not shown) also revealed normal SMFs in these transfected myotubes.

To further delineate the spatial relationships between the incorporation of these MYC/modules and their failure to bind along the ~1.0-μm polarized thin filaments two double staining experiments were performed. Day 10 MYC/M160-M183-transfected cultures were stained with anti-MYC and mA bs to either cardiac- or skeletal α-actin (anti-c- or anti-s-α-actin), or (b) anti-MYC and Rho-phalloidin. For reasons not yet understood, the mA bs to both F-α-actins do not localize within the Z-bands proper, either in normal developing (Lin et al., 1987, 1998) or adult (Otey et al., 1988) SMFs. Rather, in both developing and adult SMFs, the anti-α-actins continuously decorate regions of the polarized I-band filaments up to, but not including, the Z-bands. Similarly, in double-stained MYC/M160-M183-transfected myotubes, while the α-antisera localize along part of the ~1.0-μm I-band complexes, they are excluded from the Z-band proper (Fig. 3, D–D’; see similar pattern in SMFs in cultured cardiomyocytes in Handel et al., 1991). Failure of the mA bs to α-actin to decorate the entire thin filament, particularly the intra-Z-band, F-α-actin is not likely due to steric hindrance owing to the size of the IgG molecules. For antibodies to s-α-actinin, nebulin (SH3), and titin (Z1Z2) double stain intra-Z-band components. It will be important to understand why the anti-α-actins fail to bind to the intra-Z-band F-α-actin oligomers (Yamaguchi et al., 1985), but bind those elsewhere in the thin filament complexes (Lin et al., 1998).

While FITC- and Rhophalloidin are invaluable for detecting F-actin in cell-free systems and in a variety of cytoskeletal structures in nonmuscle cells, their binding to F-α-actin in ~1.0-μm thin filament complexes is not well understood. Under a variety of conditions (e.g., tension, rigor, presence of nebulin, sarcomere length, time in stain, concentration, etc.), neither phalloidin stains the ~1.0-μm I-band filaments uniformly. Rather, they bind to multiple bands per sarcomere (Bukatina et al., 1984; Antin et al., 1986; Wilson et al., 1986; A o and Lehrer, 1995; Y asuda et al., 1995; Zhukarev et al., 1997). As illustrated in Figs. 3 (E–E’) and 4 (A–D), while anti-s-α-actinin and anti-MYC localized uniquely in Z-discs, FITC- or Rho-phalloidin stains three separate bands/sarcomere. One band coincides with the narrow Z-discs, the other broader bands are limited to the distal ends of the thin filaments. Whereas the phalloidins bind to the intra-Z-band F-actin oligomers, others that stretch along the length of the ~1.0-μm thin filament are either relatively inaccessible, differentially quenched or enhanced, or have a higher off-rate to the F-actin staining reagents (see Materials and Methods and Discussion). Whether the discontinuity of phalloidin staining at sites along the length of I-band thin filaments is due to local structural inhomogeneities, or local reconfigurations in F-α-actin induced by other myofibrillar molecules (e.g., A o and Lehrer, 1995; Nagaoka et al., 1995; McCough et al., 1997), has yet to be determined.

In summary, in contrast to data on the actin-binding properties of nebulin modules (regions M9–M179; Wang et al., 1996; Gonsior et al., 1998; Zhang et al., 1998) which bind and polymerize F-actin filaments in vitro, binding of MYC/M160-M183 or MYC/M160-M175 to polarized F-α-actin I-band filaments was not observed in developing myotubes. Both nebulin fragments were incorporated exclusively into periodic 0.1-μm Z-bands. If they behaved as dominant negatives or were cytotoxic (see below), then these effects could not be detected microscopically. It is worth noting that similar findings were observed for most of the MYC/s-α-actinin fragments, the other major intra-Z-band actin-binding protein (Schultheiss et al., 1992; Lin et al., 1998; Ojima et al., 1999).

Localization of MYC/M171-M183

The temporospatial incorporation of MYC/M171-M183 (Fig. 1) differed from that of MYC/M160-M183 and MYC/M160-M175. Equally important, the fate of this particular MYC/nebulin peptide differed in day 4–6 versus day 10...
myotubes. It was not incorporated selectively into Z-bands in the younger myotubes, but was localized in ectopic fine granules distributed throughout the sarcoplasm and in ectopic longitudinal fibrils/patches (Fig. 5, A–C'). The MYC/granules are probably self-aggregates since they do not co-stain with Rho-phalloidin, or with antibodies to any of the I-Z-I proteins tested (see below). Comparable diffuse sarcoplasmic granules positive for endogenous nebulin (e.g., positive for antinebulin NB2) are never observed in nontransfected postmitotic myoblasts or immature or mature myotubes (Lin et al., 1994, 1998; Ojima et al., 1999). The ectopic MYC-positive longitudinal fibrils/patches, which are nested among normal individual SMFs, range in length from 5 to 200 μm, and in width from 0.3 to 5 μm. On the other hand, as shown in Fig. 5 C, most ectopic fibrils/patches costain for both exogenous and endogenous nebulin peptides. Table I summarizes the results of double staining the ectopic fibrils/patches with anti-MYC and antibodies and reagents to other I-Z-I proteins. In contrast to the MYC/granules, most MYC-ectopic fibrils/patches are positive for phalloidin, anti-c-α-actin, antinebulin NB2, and, to a lesser extent, tropomyosin. These ectopic structures are readily distinguished from precursor I-Z-I bodies, for close to 100% of the precursor myofibrillar structures stain discontinuously for titin, s-α-actinin, T-cap, and nebulin (Lin et al., 1998; Ojima et al., 1999). Whether fibrils and patches differ in more subtle details remains to be determined. Of particular interest...
was that between days 4 and 10, there was a progressive diminution in both ectopic MYC/granules and fibrils/patches. Conversely, during this period, MYC/Z-bands became more prominent (Fig. 5, D–D'). This striking change in localization over time of expressed MYC/M171-M183 was not observed with any of the other MYC/nebulin peptides shown in Fig. 1.

In summary, MYC/M171-M183 was not specifically incorporated into Z-bands in day 4 myotubes, but was incorporated into diffuse granules and fibrils/patches. The loss of these ectopic structures during days 4–6, coupled to the progressive emergence of MYC/Z-bands in day 10 myotubes, suggests the mediation, in older myotubes, of unknown regulatory mechanisms designed to cope with misfolded peptides and/or ectopic structures. Interestingly, Ojima et al. (1999) reported that misoriented ectopic preTABLE I. Variable Composition of Double-stained MYC/nebulin Ectopic Fibrils/Patches

| Protein                  | MYC-positive | Rho-phalloidin-positive | c-α-actinin-positive | Nebulin (NB2)-positive | Tropomyosin-positive | S-α-actinin-positive | Titin (Z1Z2)-positive | MHC-positive |
|--------------------------|--------------|-------------------------|---------------------|------------------------|----------------------|----------------------|-----------------------|--------------|
| 100% (n = 210)           | 93% (n = 30) | 67% (n = 30)            | 60% (n = 30)        | 37% (n = 30)           | 3% (n = 30)          | 0% (n = 30)          | 0% (n = 30)           |

Day 10 cultures expressing MYC/M171-M183 were double stained with anti-MYC and Rho-phalloidin, or with antibodies to other myofibrillar proteins. A total of 210 randomly selected MYC-positive fibrils/patches were examined, and the percentage that costained for the other myofibrillar proteins determined.
cursor I-Z-I bodies positive for s-α-actinin, titin, tropomyosin, and troponin were also resorbed in older myotubes. Localization of MYC/M175-M184

MYC/M175-M184 peptides (Fig. 1) were not incorporated selectively into mature Z-bands at any time. They were primarily incorporated into fine sarcoplasmic granules, fibrils/patches, and occasionally ill-defined striations (Fig. 5, E–E '). In most respects, they were similar to MYC/M 185-Ser (see below). The widespread ectopic MYC-positive structures did not interfere with the assembly of morphologically normal periodic Z-bands (A'). Arrows point to lateral sarcoplasmic extensions that are not occupied by SMFs. (B–B') Day 6 MYC/M 171-M 183-transfected culture, triple stained as above. In addition to the ectopic MYC-positive granules, note the ectopic slender nonstriated longitudinal filaments (arrows) that insert between normal SMFs (see below). Neither ectopic MYC-positive structure binds anti-sα-actinin, nor does it act as a dominant negative. Occasionally, just perceptible MYC/Z-bands can be observed. As discussed in Materials and Methods, these MYC/Z-bands are not likely to be due to bleed-through. A sterisks mark untransfected myotube. Day 6 MYC/M 171-M 183-transfected culture, triple stained with (C) antinebulin (NB2), and (C') DAPI. Antinebulin (NB2) localizes to the endogenous nebulin. Note that ~70% of the ectopic MYC-positive filaments/patches costain with antinebulin (NB2). A nti-MYC, but not antinebulin (NB2), accumulates along the edge of the lateral sarcoplasmic extensions (arrows), which lack all myofibrillar structures. MYC/Z-bands are more evident in this day 6 myotube than in younger myotube in Fig. 5 A. A sterisks mark two out-of-focus, overlapping untransfected myotubes. Day 10 MYC/M 171-M 183-transfected culture, triple stained with (D) anti-MYC, (D') anti-sα-actinin, and (D') DAPI. Note the elimination of the ectopic MYC-positive granules and filaments/patches in these older myotubes. A sterisks mark out-of-focus untransfected myotubes. Arrow points to an out-of-focus binucleated myotube. Day 4 myotubes expressing MYC/M 175-M 184. Triple stained with (E) anti-MYC (E'), anti-MHC, and (E') DAPI. Ectopic MYC-positive granules and fibrils/patches dominated these transfected cells. MYC-positive Z-bands were not evident. Nevertheless, the morphology of the 1.6-μm A-bands (E') was normal. A sterisks mark untransfected myotube. Bar, 10 μm.

Localization of MYC/M175-M184

MYC/M 175-M 184 peptides (Fig. 1) were not incorporated selectively into mature Z-bands at any time. They were primarily incorporated into fine sarcoplasmic granules, fibrils/patches, and occasionally ill-defined striations (Fig. 5, E–E '). In most respects, they were similar to MYC/M 185-Ser (see below). The widespread ectopic MYC-positive structures did not interfere with the assembly of normal SMFs. They did not behave in a dominant negative manner, nor were they obviously cytotoxic, even in day 10 myotubes. Localization of MYC/M175-SH3

MYC/M 175-SH3 (Fig. 1) was incorporated into Z-bands in day 4–10-transfected myotubes. Its behavior differed from that of MYC/M 160-M 183 only in that, occasionally, it was also incorporated into ectopic MYC/fibrils/patches. This
MYC/peptide was not incorporated into ectopic MYC-positive granules nor into ~1.0-μm F-α-actin thin filament complexes (Fig. 6, A–C’).

Localization of MYC/M175-Ser and MYC/M185-SH3

While being incorporated primarily into diffuse granules and fibrils/patches, both MYC/M175-Ser and MYC/M185-SH3 were also weakly localized in morphologically normal Z-bands (Fig. 6, D–E’’, and Fig. 7, A–A’’). This applied to day 4–10 myotubes. Most frequently, however, the fluorescent intensity of these MYC/Z-bands was modest compared with that of MYC/M160-M183 (Fig. 3, A–E’’) or of MYC/M175-SH3 (Fig. 6, A–C’’). In no instance did either of these MYC/nebulin peptides bind continuously along ~1.0-μm thin filament complexes nor did they behave in a dominant negative fashion.

Localization of MYC/M185-Ser

MYC/M185-Ser peptides were not selectively incorporated into Z-discs in early or late myotubes, but accumulated diffusely throughout the sarcoplasm (Fig. 7, B–C’’). Not infrequently, they displayed a variety of poorly defined cross-banded structures. Their presence did not perceptibly block normal myofibrillogenesis nor were they detectably cytotoxic in myotubes (see below).

Localization MYC/- or GFP/M86-M92

The distribution of MYC/- or GFP/M86-M92 (Fig. 1) also
demonstrates that the incorporation of nebulin modules into their site-specific locations in I-Z-I bands requires conditions not yet defined. M 86-M 92 constitutes the 12th super-repeat of nebulin and in situ is postulated to be located roughly in the middle of \( z_{1.0-}\)mF-actin thin filament complex (Pfuhl et al., 1996). Both the MYC- and the GFP-peptides localized to ectopic granules and fibrils/patches in day 4–10-transfected myotubes (Fig. 7, D and D’). The overall distribution of these tagged fragments was not readily distinguishable from that of COOH-terminal MYC/M 175-M 184 and MYC/M 185-Ser. They were not incorporated into \( \sim 1.0-\)m thin filament complexes nor were they dominant negatives. The distribution of the 10 tagged nebulin fragments, described in this report, is summarized in Fig. 8.

In Vitro Binding Properties of the Nebulin COOH-terminal SH3 Domain

Recently, high affinity in vitro binding of \( \alpha \)-actinin and actin to the SH 3 domain of nebullette was described (M onc-

man and Wang, 1999). On the sequence level, the nebullette and nebulin SH 3 domains share 80% sequence identity (Politou et al., 1998), suggesting that the COOH-terminal SH 3 domains of nebulin in skeletal muscles and nebullette in heart muscles might be involved in the same protein interactions. To test this, we performed in vitro experiments with nebulin SH 3 from human skeletal muscle, and purified F-actin and \( \alpha \)-actinin from rabbit muscle. The binding of SH 3 to F-actin and \( \alpha \)-actinin was studied by sedimenting F-actin in the presence of nebulin SH 3 and in the presence of nebulin SH 3 together with actin and s-\( \alpha \)-actinin. Samples of pellets and supernatants were analyzed on 4–20% SDS gels. When F-actin was sedimented in the presence of nebulin SH 3, the nebulin SH 3 remained in solution (Fig. 9). This indicates that nebulin SH 3 does not bind to F-actin in this cell-free system. To examine whether nebulin SH 3 binds to s-\( \alpha \)-actinin, a mixture of all three proteins was centrifuged. As seen in Fig. 9, F-actin and \( \alpha \)-actinin coprecipitated in a 1:1 ratio, while nebulin was found in the supernatant. Thus, we conclude that the SH 3 domain does not bind to actin or \( \alpha \)-actinin under the conditions used.
All Nebulin Modules Are Cytotoxic in 72–96-h Posttransfected Mononucleated Cells

Lin et al. (1998) and Ojima et al. (1999) reported that, in myogenic cultures, MYC/s-α-actinin was cytotoxic in most 72–96-h posttransfected mononucleated cells. This applied to both replicating fibroblasts and precursor myogenic cells. In contrast, the same MYC/s-α-actinin fragments were not cytotoxic, even when expressed for as long as 10 d in myotubes. Comparable differences in cytotoxicity between mononucleated cells and myotubes obtain in cultures expressing any of the 10 tagged-nebulin fragments. Roughly 80% of the nebulin-transfected mononucleated cells in day 4 cultures displayed the same moribund cytoskeleton syndrome displayed by cells expressing MYC/s-α-actinin peptides, namely intensely fluorescent nuclei and cytoplasmic filamentation, as well as resorption of all cytoskeletal elements including adhesion plaques (Fig. 10, A and B). The frequency of such moribund cells diminished greatly by day 6, and by day 10, was very rare. Hijikata et al. (1997) also reported that MYC/s-α-actinin was cytotoxic in most 72–96-h posttransfected PtK2 cells. Whether the cytotoxic effects of both nebulin and s-α-actinin fragments in mononucleated cells reflects ratios of exogenous peptides/cytoskeletal elements or in mononucleated cells to that in myotubes, or reflects differences in how myotubes cope with aberrant myofibrillar molecules versus how mononucleated cells cope, requires further experiments.

Discussion

The salient observations in this report are as follows. First, there are variations in the capacity of COOH-terminal nebulin modules that incorporated exclusively into Z-bands versus those incorporated ectopically into fine sarcoplasmic granules and fibrils/patches. Second, contrary to the strong binding of nebulin modules to purified F-actin in vitro (Root and Wang, 1994; Pfuhl et al., 1994, 1996; Zhang et al., 1998; Gonsior et al., 1998), the nebulin modules used in this report did not differentially bind to ~1.0-μm F-actin thin filament complexes in maturing skeletal myotubes. Third, there are unexpected differences between the visible distribution of F-α-actin in I-Z-I bands after decoration with anti-α-actin versus that after decoration with phalloidin. Fourth, temporospatial differences between sites of incorporation of MYC/M171-M183 in day 4 versus day 10 myotubes (Fig. 5) probably reflect muscle-specific control mechanisms (Wolff et al., 1992; Dhawan et al., 1991) responsible for the turnover of ectopic structures and/or misfolded peptides. Fifth, neither the COOH-terminal Ser nor SH3 domains are obligatory for the incorporation of a subset of nebulin fragments into morphologically normal Z-bands. Finally, MYC/nebulin or GFP/nebulin fragments, which are obviously cytotoxic in day 3–4-monomonucleated cells, are not obviously cytotoxic in day 10 maturing myotubes.

Z-bands consisting of α-actin oligomers, s-α-actinin, and T-cap molecules, as well as of NH₂-terminal titin and COOH-terminal nebulin residues, have evolved to yield a structure that resists deformation but is essential for force transmission between sarcomeres and for maintaining A-bands in the center of each sarcomere (Horowits, 1999). In such a structure, it might be expected that the stoichiometries and topological interconnections between the proteins would be fixed, rigorously controlled, and that tolerance for the incorporation of misfolded exogenous molecules be minimal. This model of precision crafted, hard-wired interdigitating filaments is consistent with the supposition that both F-α-actin binding proteins, s-α-actinin and nebulin, independently and/or cooperatively, are probably involved in regulating the incorporation and turnover of intra-Z-band F-α-actin. Alternatively, I-Z-I bands may be more compliant and dynamic structures (Schroeter et al., 1996), rich in unsaturated binding sites, designed to exchange ligands rapidly. Glycerinated day 100-102 skeletal myotubes
4–10 myotubes rapidly bind exogenous heavy meromyosin monomers, forming myriad polarized arrowheads uniformly distributed along the entire length of in situ ~1.0–
μm F-α-actin thin filament complexes (Ishikawa et al., 1969; Ojima et al., 1999). Similarly, fluorescein-labeled actin, or α-actinin, microinjected into living myogenic cells is promptly incorporated into preexisting I-Z-I structures (Glacy, 1983; Mekenna et al., 1985; Sanger et al., 1986). Kunst et al. (2000) reported His6-tagged phosphorylated and unphosphorylated M yB-P-C fragments diffused into skinned cardiac myocytes and selectively accumulated in A-bands. Finding that M Y C fragments of both s-α-actinin (Schultheiss et al., 1992; Holtzer et al., 1997; Lin et al., 1998; Ojima et al., 1999) and nebulin are incorporated uniquely into Z-bands further supports a model rich in unsaturated binding sites, as well as one unexpectedly tolerant of incorporating some types of aberrant myofibrillar peptides. In developing Drosophila muscles, exogenous headless myosin coassembles with endogenous myosin to form thick filaments (Standiford et al., 1997; Cripps et al., 1999). Similarly in dystrophin-deficient mdx/mice truncated utrophin inserts, not just in the sarcolemma subjacent to its normal location in the myoneural junction but displays an ectopic subsarcolemmal global distribution (Tinsley et al., 1996), and dystrophin fragments can be incorporated into a morphologically normal sarcolemma and mediate the assembly of the dystrophin-associated glycoprotein complex (Lu et al., 2000). Whether SMFs with M Y C/Z-bands function properly or in myotubes older than day 10 would prove to be a dominant negative and induce nemaline-like bodies (e.g., Schultheiss et al., 1992; Lin et al., 1998) or be cytotoxic, must still be determined.

Assuming that nebulin modules 177–185 plus the Ser and SH3 domains are integral to Z-bands, whereas the more NH2-terminal modules are integrated uniformly into the polarized ~1.0–μm thin filaments, then normally only 5–7% of the length of nebulin is confined to the Z-lattice. It will be interesting to determine whether the four M Y C/ COOH-terminal fragments that are incorporated exclusively into Z-bands in myotubes will bind to F-actin, as well as to other integral Z-band peptides in cell free systems and whether any modules NH2-terminal to M 160-M 175 are incorporated selectively into Z-bands. Alternatively, the intracellular integration into Z-bands of a subset of COOH-terminal nebulin peptides may require the concurrent and cooperative interactions of several Z-band components, or even of assemblases (Barral and Epstein, 1999), as has been postulated for the integration of s-α-actinin fragments into Z-bands (Ojima et al., 1999).

Our findings do not exclude the possibility of favorable electrostatic conditions that permit side-by-side binding of positively charged nebulin modules to the negatively charged surface of intra-Z-band F-α-actin oligomers (Amann et al., 1998), or the groove near the phalloidin binding site at the center of the actin α-helix. Zhang et al. (1998) and Gonsior et al. (1998) reported that despite their insolubility, some nebulin modules could be incorporated into F-actin and cosediment in a cell-free system. Microscopically, such in vitro reconstituted F-actin/nebulin aggregates resemble the ectopic fibrils/patches assembled in myotubes (Fig. 5, B and C, Fig. 6, A and E, and Fig. 7 A). Recently, comparable findings have been observed with different titin N2A fragments. Ojima, K., Z. Lim, and H. Holtzer (unpublished data) found that the incorporation of titin GFP/I80-I81 was tightly regulated because it incorporated exclusively at the same level in the I-bands as that occupied by the equivalent residues of the in situ wild-type titin. In contrast, titin GFP/I76-I79 and GFP/PEVK were distributed diffusely throughout the entire sarcoplasm. Whether the spatially appropriate incorporation of fragments of nebulin or titin into SMFs involves a novel type of dimerization with their respective in situ full-length amino acid residues must be investigated. This type of modular exchange might be limited to the turnover of exceptionally long proteins with reiterative receptors.

The presence of homologous SH3 and Ser domains in the COOH termini of both nebulin and nebulette has prompted speculations regarding their role in the assembly of I-Z-I bands (Millevoi et al., 1998; Politou et al., 1998; Young et al., 1998; Moncman and Wang, 1999). However, as summarized in Fig. 8, M 160-M 183 and M 160-M 175, which lack both SH3 and Ser domains have the capacity to be incorporated into Z-bands. Thus far, we have
not identified any single site that is obligatory for the incorporation of nebulin fragments into Z-bands.

Recently, Moncman and Wang (1999) studied the effects of expressing nebulette modules in cultured-mono-nucleated cardiomyocytes. While some of our findings are in agreement with some of their conclusions, many are not. Several reasons could account for these differences. First, the method of isolating the SH3 domains differed in the two studies (see Materials and Methods). Second, there was ambiguity regarding the sites of incorporation of the expressed nebulette in the cardiac I-Z-I bands, based on phalloidin to identify actin; and third, despite the sequence similarities of the COOH-terminal nebulin and nebulette, the functions of these regions in the assembly and maintenance of the I-Z-I bands in the two phenotypes may be less conserved. In our opinion, however, the most likely explanation derives from a common misunderstanding of the behavior of the already differentiated cardiomyocytes in culture versus the behavior of presumptive skeletal myoblasts and their daughter myoblasts in culture.

Cardiomyocyte cultures are seeded with fully differentiated cells taken from the developing chick. 100% of such cardiomyocytes have already assembled thousands of contracting mature SMFs before being cultured. In contrast, skeletal myogenic cultures are seeded with mononucleated cells, none of which has assembled any myofibrillar structures. In day 4–10 cardiomyocyte cultures >90% of the SMFs previously assembled in vivo are in various stages of degeneration: only a minute fraction of the myofibrillar structures in these cells is in some stage of assembly (Dlugosz et al., 1984; Lin et al., 1989; Handel et al., 1991; Lu et al., 1992). In contrast, >98% of the myotubes in day 4–10 cultures of skeletal myogenic cells are rich in thousands of de novo assembled stable, invariant, mature SMFs (Holtzer et al., 1972, 1997; Lin et al., 1987, 1998; Fig. 2). The extent of massive degeneration of the preformed SMFs in cultures of differentiated cardiomyocytes has been grossly underestimated in the literature (e.g., Lin et al., 1989; Schultheiss et al., 1990; Lu et al., 1992; Messeri et al., 1993; Simpson et al., 1996; Sussman et al., 1997; Linke et al., 1999). In brief, the global degeneration, attributed by Moncman and Wang (1999) to the dominant negative effects of exogenous nebulin modules, may rather be the result of the spontaneous fragmentation of SMFs, which is characteristic of both untransfected and transfected cultured cardiomyocytes (e.g., compare the virtual identity of micrographs in the untransfected cardiomyocytes in Lin et al. (1989) with the transfected cardiomyocytes in Moncman and Wang, 1999). Much is yet to be learned of both the similarities, but particularly of the differences, between myofibrillogenesis in cardiac versus skeletal muscle.

The emergence of MY C/Z-bands in day 10 MY C/M 171-M 183 myotubes, which is correlated with the progressive loss of ectopic MY C structures in day 4–6 myotubes (Fig. 5, A–D), suggests selective turnover and/or posttranslational changes that would be missed in the absence of time-dependent studies. The simplest explanation is that, despite their impaired conformation, the increased concentration reaches a critical level over time, which permits these particular modules to out compete wild-type ligands for incorporation into morphologically normal Z-bands; alternatively, the ectopic MY C/M 171-M 183 structures were selectively degraded. Precedent for the selective elimination of ectopic myofibrillar structures has been described recently. M is aligned I-Z-I bodies, which consist of linear aggregates of α-actinin/nebulin/titin/α-actin/T-cap, are resolved within 24–48 h in the giant growth tips of elongating myotubes (details in Ojima et al., 1999). Despite differences in morphology and molecular composition, the selective resorption of ectopic MY C/α-actinin I-Z-I bodies, and MY C/nebulin granules or fibrils/patches, might involve similar surveillance systems. Until more is known about the kinetics of the turnover of full-length nebulin in living myotubes, as well as of those of the other integral Z-band proteins, speculations about how nebulin modules might be integrated into, or excluded from, normal Z-band structures, or how they are resolved from ectopic structures, are premature. By following the fate of GFP/nebulin modules over time (e.g., 4–30 d; Holtzer et al., 1972) in living myotubes, we should be able to determine the following: whether their incorporation is regulated by the same mechanisms that control normal turnover; whether the exogenous modules fully displace the wild-type nebulin over time, or only their COOH termini, and whether such incorporation feeds back on the regulation of either the translation or transcription of the gene for endogenous nebulin.

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