Interactions between Nebulin-like Motifs and Thin Filament Regulatory Proteins*

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Nebulin (600–900 kDa) and nebullette (107–109 kDa) are two homologous thin filament-associated proteins in skeletal and cardiac muscles, respectively. Both proteins are capped with a unique region at the amino terminus as well as a serine-rich linker domain and SH3 domains at the COOH terminus. Their significant size difference is attributed to the length of the central region wherein both proteins are primarily composed of ~35 amino acid repeats termed nebulin-like repeats or motifs. These motifs are marked by a conserved SXXXY sequence and high affinity binding to F-actin. To further characterize the effects that nebulin-like proteins may have on the striated muscle thin filament, we have cloned, expressed, and purified a five-motif chicken nebulin fragment and tested its interaction with the tropomyosin-troponin T complex. Both tropomyosin and troponin T individually bound the nebulin fragment, indicating an interaction site for the nebulin-like motifs from both nebulin and nebullette associated with F-actin. Additional experiments using in vitro systems have formed a consensus that nebulin-like motifs interact with tropomyosin (Tm) and troponin (Tn) along the striated muscle thin filament.

At the Z-line, the nebulin/nebullette interactions are probably mediated by contributions from the linker domain as well as interactions of the SH3 domains with α-actinin (6, 9). Results from Ojima and coworkers (10) suggest that neither the serine-rich linker domain nor the SH3 domain are obligatory elements for the incorporation of nebulin and presumably nebullette into the Z-line of striated muscle sarcomeres. Although both proteins are anchored at the Z-line in vivo, the primary sequence regions responsible for this interaction remain unclear. Recent experiments have also shown an association between the NH2 terminus of nebulin and tropomodulin, a protein known to cap actin-Tm filaments (11). Therefore, emerging data demonstrate that the NH2- and COOH-terminal domains of nebulin and nebullette form unique interactions at both the pointed end of the thin filament and the Z-line of sarcomeres, complementing the established actin binding properties of these proteins.

Although the majority of experiments to date have focused on the binary interactions between nebulin/nebullette and other sarcomeric proteins, there is preliminary evidence to suggest that nebulin-like motifs may serve a role in the regulation of striated muscle contraction. These investigations have concentrated on the role that nebulin may play in regulating interactions within the framework of the isolated acto-S1 myosin system. Consistent with this hypothesis, it has been demonstrated that nebulin fragments of varying lengths are able to inhibit the acto-S1 myosin ATPase, an inhibition that can be reversed by calmodulin (12). Subsequent experiments by this group have suggested that nebulin fragments affect the acto-S1 myosin complex by optimizing the alignment of actomyosin interactions (13). To further clarify the interactions that nebulin-like repeats may have along the thin filament, we have characterized the binding of a chicken nebulin fragment to Tm and the Tn subunits. Our data suggest that Tm and tropomin T (TnT) both contain interaction sites for the nebulin-like motifs. These interactions were strengthened when Tm and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF440239.‡ To whom correspondence should be addressed. Tel.: 216-368-5525; Fax: 216-368-3952; E-mail: jxj12@po.cwru.edu.

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1 The abbreviations used are: Tm, tropomyosin; CN5, 5-motif chicken nebulin fragment; mAb, monoclonal antibody; MSN, 4-motif mouse nebulin fragment; RT, reverse transcription; Tn, troponin; TnC, tropomin C; TnI, troponin I; TnT, troponin T; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); kb, kilobase; SSPE, saline/sodium phosphate/EDTA.
TNt were present as a binary complex and weakened by the addition of tropinin I (TnI). Furthermore, the nebulin fragment was able to significantly increase the affinity of TNt for F-actin, demonstrating that nebulin-like motifs contribute to the assembly and allosteric properties of the striated muscle thin filament.

**EXPERIMENTAL PROCEDURES**

Reverse Transcription (RT)-PCR Cloning of Mouse Nebulin and Chicken Nebullet—To clone mouse nebulin (MSN) and chicken nebullet (CN5) cDNAs, a partial sequence of nebulin cDNA from BALB/c strain mice (14) and a partial cDNA sequence from chicken was used to design primers suitable for RT-coupled PCR. Total RNA was prepared from 129 Sv/j mouse gastrocnemius muscle or white Leghorn chicken heart by the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Using 5 μg of total RNA as template and 20 pmol of a primer reverse (mouse: Neb-3′, 5′-CGGCATCATGCTTTTCTTGCATAC-3′, and chicken: CCN-R2, 5′-CCTTTGAATCCAAATTCTTCAAGT-3′) in 50 μl of reaction solution, and 100 g/ml denatured salmon sperm DNA at 50 °C overnight. The hybridization and washing conditions were identical to those described herein. The hybridized filters were washed successively at room temperature with 2×SSC followed by washing with 0.1×SSC and 0.1×SSPE at 60 °C for 1 h. The filters were exposed to x-ray film overnight to reveal hybridization signals. A small agar plug was recovered from the plate corresponding to the area of each putative positive signal. The hybridization screening was then used in plaque purification to isolate the positive phage clones.

The positive phage DNA was prepared in large quantity using the Qiagen Lambda Midi Kit according to the manufacturer’s instructions (Qiagen). An ~15-kb mouse genomic DNA insert was excised from the λ-phage arms by EcoRI digestion, and the EcoRI fragments were subcloned into the EcoRI site of pBluescript SK II vector. One subclone containing a mouse genomic DNA fragment was sequenced by preparing a set of deletions using an exonuclease III-based nested deletion kit according to the manufacturer’s instructions (Promega).

**Protein Expression and Purification—Escherichia coli BL21(DE3)—** pLysS cells were transformed with the T7 polymerase-based pAED4-CN5 chicken nebullet expression construct and allowed to grow until the colonies were just visible. Four fresh colonies were randomly picked to inoculate 4 liters of NZ broth (10 g/liter casein hydrolysate, and 5 g/liter NaCl) containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. The cultures were incubated overnight at 37 °C with shaking until A600 = 0.8 at which time protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.4 mM. Following 3 h of growth, the bacteria were harvested by centrifugation and lysed in 20 mM imidazole, pH 7.0, and 1 mM EDTA by three passes through a French press at 1000 p.s.i. Successive ammonium sulfate cuts from 0 to 30% saturation and from 30 to 50% saturation were taken from the cleared bacterial lysate. The pH of the solution was maintained at 7 throughout the ammonium sulfate precipitation procedure. The 30% solution (ammonium sulfate pellet) was reapplied and precipitated with 20 mM imidazole, pH 7.0, 1 mM EDTA, and 6 mM β-mercaptoethanol and dialyzed at 4 °C against 4 liters of 20 mM imidazole, pH 7.0, 1 mM EDTA, and 6 mM β-mercaptoethanol for two changes. To the diazoyed fraction, urea powder was added to 6 M, the pH was adjusted to 7, and the solution was clarified by centrifugation. Urea was used to minimize protein-protein interactions and maximize separation during ion-exchange chromatography. The supernatant was loaded onto a 100-ml (2.5 × 20 cm) CM52 column equilibrated with 6 M urea, 20 mM imidazole, pH 7.0, 1 mM EDTA, and 15 mM β-mercaptoethanol. A linear KCl gradient of 0–400 mM in the equilibration buffer was used to elute the CN5 protein from the column. The fractions from the CM52 column were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the authenticity of the protein of interest were collected, dialyzed against 4 liters of 0.1% formic acid, a volatile agent, for three changes, and lyophilized. Amino acid analysis of the purified CN5 protein verified the authenticity of cDNA expression and the effectiveness of protein purification (Molecular Biology Core Laboratory, Case Western Reserve University, Cleveland, OH).

The purification protocols for TNc, Ti, TnI, and α/β-Tn from chicken skeletal muscle have been described previously, and the purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18). Concentrations for all protein preparations were determined by the NanoProtein Plus Assay Kit (Bio-Rad). The purity of these preparations have been demonstrated (17, 18).
to produce high titer supernatant and introduced into 2,6,10,14-tetramethyl pentadecane (Pristane, Sigma) primed peritoneal cavity of BALB/c mice to produce mAb-enriched ascites fluids. The hybridoma culture supernatant was used in this study.

Western Blot Identification of Nebulette—To monitor nebullet expression, Western blots were done using either the anti-CN5 antiserum or CN5 mAb Ne-110 on fresh SDS extracts of cardiac muscle from various species. The SDS-PAGE samples were prepared in the presence of 1 mM phenylmethylsulfonyl fluoride and 20 μM leupeptin (Sigma) to ensure protein integrity. To better resolve large proteins (>100 kDa), 6% SDS-PAGE with an acrylamide:bisacrylamide ratio of 29:1 was used. However, in this system, the resolving gel does not retain proteins <60 kDa in molecular mass. To ensure that no proteolytic products <60 kDa in molecular mass were present, 14% (180:1) SDS-PAGE was used to present a complete SDS extract profile. After resolving total cardiac muscle extracts by SDS-PAGE, a three-buffer system was used to efficiently transfer the resolved protein bands onto nitrocellulose membranes (21). Following the transfer, the nitrocellulose replicas were blocked by incubation at room temperature for 3 h with Tris-buffered saline (150 mM NaCl, 2.5 mM KCl, and 25 mM Tris-HCl, pH 7.5) containing 1% bovine serum albumin. The blocked membrane was then incubated at 4 °C overnight with either the anti-CN5 antiserum or Ne-110 mAb diluted in Tris-buffered saline containing 0.1% bovine serum albumin. The Western blots were developed via alkaline phosphatase-labeled secondary antibody as described previously (17).

Nebulette Affinity Chromatography—The relative binding strengths of α-Tm and Tn subunits to the chicken nebullet fragment CN5 were determined using affinity chromatography. The purified nebullet fragment was coupled to CNBr-activated Sepharose 4B gel (Amersham Biosciences) according to the manufacturer’s instructions using 9 mg of CN5 protein/0.5 ml of gel for coupling. A 0.4-ml column was packed and equilibrated with 100 mM NaCl, 15 mM PIPES, pH 7.0, 3 mM MgCl₂, 1 mM K₂EGTA, and 0.1 mM tris(carboxymethyl)phosphate. Proteins to be analyzed on the column were dissolved in 3 ml of the column buffer and dialyzed overnight at 4 °C against 2 liters of the column buffer to ensure homogenous buffer conditions and complex formation. For binding experiments that test the interaction of α-Tm or TnT with the CN5 affinity column, 0.4 nmol of protein was typically used. For experiments involving complexes of Tm with Tn subunits, 0.4 nmol of Tm dimer was used and the Tn subunits were mixed in a 1:1 molar ratio. After loading the diazed protein mixtures, the column was washed with 8 bed volumes of the equilibration buffer and eluted by a step gradient of 120–680 mM NaCl in the equilibration buffer. All of the steps were carried out at room temperature. The fractions were examined by 12% SDS-PAGE and visualized by silver staining to determine the protein peaks (22). A control experiment was done by mixing 0.4 nmol of TnT and bovine serum albumin (New England Biolabs) and loading onto the CN5 affinity column. The TnT bound specifically to the column since the bovine serum albumin was eluted in the flow-through and wash fractions only, indicating that nonspecific interactions in this setting were minimal (data not shown).

F-actin Cosedimentation—To determine the binding affinity of the CN5 nebullet fragment to F-actin and the interactions between thin filament regulatory proteins and CN5 in the presence of F-actin, a series of cosedimentation experiments were performed. Purified rabbit skeletal muscle F-actin (0.13 nmol) was incubated with serial concentrations of CN5, Tm, TnT, Tni, or whole troponin in either a 30- or 50-μl volume for 45 min at 4 °C. The cosedimentation buffer contained 10 mM potassium phosphate buffer, pH 7.4, 65 mM KCl, 2.5 mM MgCl₂, 0.1 mM K₂EGTA, and 1 mM diethiothreitol. In experiments in which CN5 was present during Tm or Tn subunit titrations, a constant concentration of CN5 nebulette fragment to F-actin and the interactions between thin filament regulatory proteins and CN5 in the presence of F-actin, a series of cosedimentation experiments were performed. Purified rabbit skeletal muscle F-actin (0.13 nmol) was incubated with serial concentrations of CN5, Tm, TnT, Tni, or whole troponin in either a 30- or 50-μl volume for 45 min at 4 °C. The cosedimentation buffer contained 10 mM potassium phosphate buffer, pH 7.4, 65 mM KCl, 2.5 mM MgCl₂, 0.1 mM K₂EGTA, and 1 mM diethiothreitol. In experiments in which CN5 was present during Tm or Tn subunit titrations, a constant concentration of CN5 was used. Following incubation, F-actin was sedimented by centrifugation at 100,000 × g (45,000 rpm) for 30 min at 4 °C in a Beckman Optima TL Ultracentrifuge using a TLA100.1 rotor. The supernatant and pellet were separated, and 15 μl of 1 × SDS-PAGE sample buffer was used to solubilize the pellet. The supernatant and pellet were examined by either 14% (180:1) or 12% (29:1) SDS-PAGE. Resolved gels were silver-stained as described before and analyzed by gel densitometry. The titration curve was constructed from results of three experiments, and the data are presented as the mean ± S.D. To estimate the binding affinity, the total [CN5] required for 50% maximum binding was obtained by averaging the data calculated from the fits for each individual experiment using single exponential fits.

RESULTS

Exon Organization of the Nebulin Gene—A mouse nebulin cDNA fragment was cloned by RT-PCR according to a published cDNA sequence (14). This cDNA was used as a probe to isolate an ~15-kb mouse nebulin genomic DNA fragment from a phage library, and an ~3.2-kb EcoRI fragment of the genomic DNA was subcloned and sequenced. The nucleotide sequence has been submitted to the GenBank Data Base (GenBank™ accession number AF440239). Two consecutive exons of the nebulin gene were found in the genomic DNA fragment encoding 69 amino acids and 35 amino acids, which correspond to two and one of the ~35 amino acid-repeating motifs, respectively (Fig. 1). Both exons had splice boundaries within the conserved pentapeptide, specifically at SXXXY, indicating this sequence to be at the boundary between the ~35 amino acid motifs. Partial sequencing of other EcoRI subclones of the ~15-kb
genomic DNA showed similar exon boundaries (data not shown). Because exon organization reflects the evolutionary and potentially functional units of multi-domain proteins, the genomic structure of the mouse nebulin gene indicates that the single ~35 amino acid motif (Fig. 1, exon B) represents the fundamental functional unit of nebulin-like proteins.

Cloning, Expression, and Purification of the CN5 Nebulette Fragment—Using oligonucleotide primers designed according to a published partial cDNA sequence (2), a chicken nebullette cDNA encoding a polypeptide beginning and ending with the conserved SXXXY pentapeptide was amplified. This frame coincides with the exon boundaries of the nebulin gene (Fig. 1). The chicken nebullette cDNA encodes a 168-amino acid protein spanning five nebulin-like motifs with a theoretical Mr of 19398 and an isoelectric point of 9.73 and is shown to be aligned to the area of highest homology within the repeating domain of human nebullette (Fig. 2) (5, 6). Similar to the human nebulin fragments as reported previously (8), CN5 expressed in E. coli at high levels. The CN5 protein was readily purified by standard biochemical procedures. Purified CN5 migrated as a single band in SDS-PAGE (Fig. 3) and was found to be relatively

![Fig. 3. Expression and purification of the chicken nebullette fragment.](image)

![Fig. 4. Expression of nebullette in cardiac muscle.](image)

![Fig. 5. Expression of nebullette isoforms during heart development and in different chambers.](image)
soluble under non-denaturing conditions (solubility >0.5 mM). This was in contrast to the MSN fragment, which was confined to the inclusion bodies upon large scale expression in E. coli (data not shown), similar to previously published data on mammalian nebulin fragments (6, 8, 24). A mouse polyclonal antiserum was generated using the purified CN5 protein as the immunogen. As shown in Fig. 3, the polyclonal antiserum was specifically identified the CN5 protein in Western blot. This finding was also recapitulated by the anti-CN5 mAb Ne-110 (data not shown).

Expression of Nebulette in Cardiac Muscle and during Development—As shown in Fig. 4, the anti-CN5 mAb strongly identified nebulin-like proteins from chicken and bovine heart extracts. The monoclonal antibody did not strongly recognize nebulin in mouse, sheep, or rabbit cardiac muscle extracts, implying epitope diversity of nebulin proteins across species. Initially, 14% (180:1) SDS-PAGE was used to resolve the isoforms to ensure that no proteolytic fragments were present. To further increase the resolution of isoforms, 6% (29:1) SDS-PAGE was used. Although the chicken nebulin isoforms were identified as two very closely migrating bands under 14% (180:1) SDS-PAGE, 6% (29:1) SDS-PAGE was better able to resolve the isoform diversity. Using the chicken as a developmental model (total extracts from embryonic days 14 to 20), neonatal and adult chicken hearts were analyzed for nebulin expression (Fig. 5). Multiple bands were identified by the anti-CN5 mAb, suggesting nebulin isoform diversity (5). A higher Mr isoform appeared to be predominant throughout heart development but was down-regulated by 6 weeks post-hatch. This developmental pattern of high to low Mr isoform expression is reflective of the regulation of TnT expression with development (23), suggesting that this type of isoform switch is fundamentally conserved in the myofilament proteins of striated muscle. Two closely migrating isoforms, a predominant lower Mr isoform and a less expressed higher Mr isoform, were identified in extracts from bovine heart chambers, suggesting that isoform diversity was not confined to the avian heart. It was also noted that the ratio of the isoforms varied between chambers with the lower Mr isoform being predominant in the left ventricle. The primary sequence differences between the nebulin isoforms are of interest but remain to be determined.

TnT Interaction with CN5 In Strengthened by Tm and Weakened by TnI—Using affinity chromatography and F-actin cosedimentation, the interactions between CN5 and the thin filament proteins F-actin, TnT, TnI, and Tm were tested. Consistent with data previously demonstrated for other nebulin fragments, CN5 specifically bound to F-actin in cosedimentation experiments with half-maximal binding occurring at 2.09 ± 0.6 μM CN5 (Fig. 6). The maximum stoichiometry of CN5 binding to uncovaled F-actin was ~0.4 mol CN5/mol actin or 2.8 mol of the 5-unit CN5 protein/T actin monomers that defines the striated muscle thin filament regulatory unit. This finding suggests that in the absence of regulatory proteins on the actin filament, the CN5 protein binds at approximately twice the stoichiometry that would be expected if each of the five nebulin motifs bound one actin monomer (1.4 mol CN5/7 mol actin). Assuming that the ~35-amino acid sequence motif represents an actin-binding unit, this 2:1 binding ratio to naked actin filaments may be a reflection of the multiple binding sites available for nebulin on each actin monomer as well as additional nonphysiological binding modes that nebulin fragments may undertake under these conditions (25). It should also be noted that the interaction between two or three of the nebulin-like motifs with adjacent actin monomers may be sufficient to sustain a stable association between CN5 and F-actin in vitro, allowing an overestimation of the stoichiometry with the unregulated actin filament. This is supported by the observation that a 3–4-unit nebulin fragment can cross-link adjacent F-actin strands (25). Although the binding between CN5 and F-actin was analyzed simply as a one-step reaction, it remains possible that the binding of nebulin and nebulin-like fragments containing multiple actin binding motifs to F-actin may be a corporative multiple-step process.

To test the direct interactions between CN5 and TnT or Tm, a CN5 affinity chromatography column was used. Purified αβ-Tm dimers only moderately interacted with the nebulin fragment as evidenced by the flow-through and subsequent run off elution from the CN5 affinity column between 120 and 160 mM NaCl (Fig. 7A). In contrast, TnT alone interacted strongly with the nebulin fragment and was eluted with a primary peak at 280–360 mM NaCl (Fig. 7B). Combining TnI and Tm in a 1:1 molar ratio resulted in both proteins binding to the column and a noticeable shift to higher affinity as compared with Tm or TnT alone (Fig. 7C). The main peak of the Tm-TnI complex was eluted at 480–560 mM NaCl. Early elution of some αβ-Tm alone was observed in Fig. 7C, corresponding to the elution of uncomplexed αβ-Tm as seen in Fig. 7A. However, the overlapping elution profiles suggest that αβ-Tm bound TnT as expected, forming a binary complex that interacted with the CN5 fragment, and remained resistant to higher salt concentrations. Troponin I significantly reduced the binding affinity between CN5 and the Tm-TnI complex, resulting in peak elution of Tm-TnITni between 280 and 400 mM NaCl (Fig. 7D) well below the elution range for Tm-TnT. The complete overlap of the Tm, TnT, and Tnl peaks under this condition demonstrates that Tnl stabilized the interaction between Tm and TnT as expected to form a Tm-TnT-Tnl complex and subsequently minimized the early elution of Tm seen in Fig. 7C. The flow-through of some Tm-TnT-Tnl complexes from the CN5 affinity column was observed in Fig. 7D, indicative of Tnl affecting the Tm-TnT-CN5 interaction. When Tnl was included to form a Tm-Tnl complex, no binding to the CN5 column was detected (data not shown).

Cosedimentation experiments verified the affinity chroma-
Fig. 7. Binding of Tm, TnT, or TnI to a CN5 affinity column. The interaction of Tm and tropinin subunits, alone or in combination, with the CN5 fragment was tested by CN5 affinity chromatography. The column profiles were analyzed by SDS-PAGE. A, α/β-Tm alone showed weak binding to the immobilized CN5 fragment, demonstrated by Tm in the flow-through (F/FT) as well as an early elution peak at 120–160 mM NaCl. B, fast TnT (fTnT) alone bound strongly to the CN5 column, because it was absent in the flow-through and showed a primary peak eluting at 280–360 mM NaCl. C, the Tm/TnT complex bound more effectively to the CN5 column, resulting in a main Tm/TnT peak eluting at 480–560 mM NaCl significantly higher than that observed for Tm or TnT alone. D, addition of fast TnI (fTnI) weakened the binding of Tm/TnT with the immobilized CN5 protein as evidenced by the elution of Tm/TnT/TnI from the affinity column between 280 and 400 mM NaCl. All SDS-PAGE was 12% gel with an acrylamide:bisacrylamide ratio of 29:1 and was silver-stained to visualize the resolved proteins.

Fig. 8, the decoration of F-actin with Tm reduced concurrent nebullet binding, possibly because of Tm constraining CN5 to binding at its physiological sites on F-actin while limiting nonphysiological interactions between CN5 and the actin filament (25). In contrast, TnT was more efficient in reducing the binding of nebullet to F-actin, suggesting that the strong interaction between nebullet and TnT weakened nebullet-actin binding. However, the Tm/TnT binary complex resulted in only a moderate effect on nebullet binding to F-actin (Fig. 8B) and provided an approximate stoichiometry of 0.7 CN5/7 actin regulatory units, or half of the theoretical stoichiometry expected if each of the five nebulin motifs bound an actin monomer (1.4 CN5/regulatory unit). This lower stoichiometry may be explained by the hypothesis that in contrast to the head-tail registered binding of Tm to F-actin, the unorganized binding of nebulin fragments would not fully decorate the actin filament. These experiments demonstrated that the Tm/TnT complex and nebullet were able to decorate F-actin simultaneously. The higher amounts of CN5 binding to Tm/TnT-decorated F-
actin versus TnT-decorated F-actin suggest relative increase in the CN5-actin affinity. This effect was confirmed by assays using the Tm-Tn/TnT complex in contrast to TnT, which more effectively decreased the binding of CN5 to F-actin (A). The addition of Tm-Tn binary complexes to the titration also demonstrated a saturable inhibitory effect. (B) addition of TnI to the Tm-TnT binary complex slightly increased the inhibitory effect on CN5 binding to F-actin.

Additional cosedimentation experiments tested the effect of CN5 on the affinity of Tm-Tn assembly onto the actin filament. Tropomyosin-troponin concentration was titrated in the presence or absence of a fixed concentration (1.52 μM) of CN5. The CN5 fragment decreased the concentration of Tm-Tn required to achieve half-maximal binding to F-actin (147.4 ± 32.1 nM without CN5 versus 70.8 ± 4.9 nM with CN5; p = 0.05 by Student’s t test) (Fig. 9), further suggesting that nebulin-like motifs participate in the striated muscle thin filament assembly.

DISCUSSION
The role of nebulin-like proteins in the sarcomere of striated muscles has been reported to be primarily a structural one. Evidence has been mounted by studies showing the interaction of nebulin or nebulette fragments with F-actin (6, 8), protein constituents of the Z-line (6, 9), and with tropomodulin at the pointed ends of the thin filaments (11). Correlations between nebulin/nebulette expression and Z-line thickness as well as nebulin protein size and actin filament length in skeletal muscle sarcomeres (5, 26) have all contributed to the large body of evidence in support of a structural role for nebulin/nebulette.

Through genomic DNA cloning, we showed in this study that the exons of the nebulin gene are arranged in a manner reflective of the ~35-amino acid nebulin-like motifs that serve as the basic functional units. The partial genomic DNA sequence data suggest that the conserved SXXXY sequence straddles the junction between adjacent exons (Fig. 1) as also suggested by Millevoi et al. (5), providing a compelling case for the SXXXY sequence serving as the boundary between the repeating nebulin-like motifs. Although the use of intact proteins remains desirable, the insolubility of intact nebulin and nebulette in physiological buffers necessitates the use of protein fragments during functional characterizations (6, 8, 24). This afforded the rationale in designing a five-motif nebulette fragment defined by the conserved pentapeptide flanking each motif (Fig. 2).
Similar to published observations with other nebulin fragments (4, 8, 24), we found that the nebulette CN5 fragment bound F-actin with affinity in the low micromolar range (Fig. 6). Although unsurprising given the homology between nebulin and nebulette, this finding confirmed that F-actin binding is a conserved function of the nebulin-like motifs. For the CN5 protein, F-actin binding is relevant because this region of nebulette localizes to actin filaments when transfected into primary cultures of cardiomyocytes (6).

We observed that the binding of CN5 and α/β-Tm to F-actin (Fig. 8A) was not mutually exclusive, although there was apparent competition consistent with both proteins binding to subdomain 1 on the actin monomer (25). However, it was evident that CN5 binding to un-decorated F-actin was at approximately twice the stoichiometry when compared with Tm, Tm-TnT, or Tm/TnT/TnI-decorated F-actin (Fig. 8). This finding suggests that decoration of naked actin filaments with nebulin-like proteins is not fully indicative of their physiological position on F-actin and that the regulatory Tm-Tn filament on F-actin is able to further constrain nebulin binding sites. In addition, the results demonstrate that both nebulin and Tm have binding domains on F-actin that are not mutually exclusive, although the cosedimentation experiments did demonstrate some overlap (Fig. 8). This recapitulates an in vitro setting in which nebulin-like motifs and Tm dimers run parallel along the F-actin filament. Using affinity chromatography, we found a weak but reproducible association between the five-motif nebulette fragment and α/β-Tm (Fig. 7A). Although this interaction was a relatively low affinity, it opened the possibility that head-to-tail-linked Tm dimers and repeating nebulin-like motifs running parallel along the actin filament may provide a higher total avidity of interaction. Because the allosteric regulation of striated muscle contraction is known to involve shifts of the position of Tm on F-actin (27, 28), nebulin-like motifs and Tm may come closer into contact or distance themselves depending on the dynamic position of Tm on the actin filament, thus allowing the nebulin-Tm association to contribute reversibly to thin filament dynamics. This model agrees with previously published data (25) demonstrating that one site of nebulin-actin interaction lies at subdomain 1 of the actin, a position believed to be occupied by tropomyosin during the blocked or resting state of the striated muscle thin filament.

In contrast to Tm, the interaction between the CN5 fragment and TnT was stronger with the peak of elution occurring at 280–360 mM NaCl by affinity chromatography (Fig. 7B). Tropinin T complexed to Tm showed even higher avidity binding to CN5 (peak at 480–560 mM NaCl) (Fig. 7C). Three possible mechanisms may underlie this observation: (a) higher avidity from the sum of two independent CN5 binding sites on Tm and TnT; (b) Tm and TnT as a binary complex possibly presenting a unique interaction pocket with increased affinity for nebulin-like motifs; or (c) the binding of Tm to TnT resulting in a conformational change in TnT, consequently increasing the binding affinity of the protein for CN5. F-actin cosedimentation results further supported the formation of a Tm-TnT binding pocket for the nebulin-like motifs. Although TnT alone caused a large decrease in the binding of nebulette to F-actin (Fig. 8A), the addition of Tm moderated the effect and allowed the assembly of a thin filament accommodating both Tm-TnT and CN5 (Fig. 8B). Additional affinity chromatography experiments demonstrated that TnT decreased the affinity of Tm-TnT for the nebulette fragment (Fig. 7D), suggesting that the interaction pocket formed by the Tm-TnT complex is either directly competed by TnT or altered by a change in the conformation of TnT upon TnT binding. In either case, these results underscore the role of the conserved COOH-terminal T2 region of TnT in mediating the interaction with CN5. These findings were verified by cosedimentation experiments in which Tm-TnT/TnI was also found to modulate CN5 binding to F-actin, similar to the results using Tm-TnT (Fig. 8B). Therefore, the association of CN5 with an F-actin-Tm-TnT filament demonstrated both a Tm-TnT and an F-actin binding component with the former being challenged moderately in the presence of TnI. The interaction between CN5 and the thin filament was extended by additional cosedimentation experiments demonstrating that the nebulin-like fragment was able to increase the affinity of Tm at subdomain 1 of F-actin (Fig. 9). This increase suggests that the documented interactions, particularly between nebulin-like motifs and Tm-TnT, provided a tangible means of increasing the affinity of the thin filament assembly for F-actin. This observation also supports the hypothesis that nebulin-like motifs are able to interact with and contribute to the regulation of the Tm-Tn assembly on the actin filament.

The data collected indicate that during the allosteric control of striated muscle contraction, a reversible interaction may occur along the thin filament between nebulin and the Tm-Tn regulatory system, affecting its interaction with F-actin. The binding between Tm-TnT and nebulin-like motifs may be the pivotal association underlying this observation and offers a role for regulation by TnC or an analogous Ca\(^{2+}\)-binding protein such as calmodulin (12). Experiments by Root and Wang (13) detailing changes in actomyosin in the presence of nebulin-like motifs may be a preliminary account of how these interactions integrate within the actin filament. The data presented in Figs. 8 and 9 necessitate future experiments in testing the acto-S1 interaction in an intact regulated filament. We propose that allosteric changes during muscle contraction due to Ca\(^{2+}\) binding to TnC and/or strong cross-bridge attachment may contribute to the cooperativity of striated muscle, possibly by allowing a reversible nebulin-nebulette interaction with the Tm-Tn complex and in turn facilitating a more pronounced propagation of the transition from the blocked to the closed or from the closed to the open states of the thin filament (29). We hypothesize that the putative effect of the nebulin-like motifs on the coordinated regulation of the thin filament may contribute to the higher cooperativity of skeletal versus cardiac muscles (30–32), a possibility that will be tested most effectively in the framework of an integrated muscle fiber.

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