Expression and Purification of Large Nebulin Fragments and Their Interaction with Actin

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ABSTRACT   cDNA clones encoding mouse skeletal muscle nebulin were expressed in *Escherichia coli* as thioredoxin fusion proteins and purified in the presence of 6 M urea. These fragments, called 7a and 8c, contain 28 and 19 of the weakly repeating ~35-residue nebulin modules, respectively. The nebulin fragments are soluble at extremely high pH, but aggregate when dialyzed to neutral pH, as assayed by centrifugation at $16,000 \times g$. However, when mixed with varying amounts of G-actin at pH 12 and then dialyzed to neutral pH, the nebulin fragments are solubilized in a concentration-dependent manner, remaining in the supernatant along with the monomeric actin. These results show that interaction with G-actin allows the separation of insoluble nebulin aggregates from soluble actin-nebulin complexes by centrifugation. We used this property to assay the incorporation of nebulin fragments into preformed actin filaments. Varying amounts of aggregated nebulin were mixed with a constant amount of F-actin at pH 7.0. The nebulin aggregates were pelleted by centrifugation at $5200 \times g$, whereas the actin filaments, including incorporated nebulin fragments, remained in the supernatant. Using this assay, we found that nebulin fragments 7a and 8c bound to actin filaments with high affinity. Immunofluorescence and electron microscopy of the actin-nebulin complexes verified that the nebulin fragments were reorganized from punctate aggregates to a filamentous form upon interaction with F-actin. In addition, we found that fragment 7a binds to F-actin with a stoichiometry of one nebulin module per actin monomer, the same stoichiometry we found in vivo. In contrast, 8c binds to F-actin with a stoichiometry of one module per two actin monomers. These data indicate that 7a can be incorporated into actin filaments to the same extent found in vivo, and suggest that shorter fragments may not bind actin filaments in the same way as the native nebulin molecule.

INTRODUCTION

Nebulin is a major component of skeletal muscle myofibrils (Wang, 1985). This unusually large, elongated protein is an integral component of the sarcomeric actin filaments in these muscles (Wang and Wright, 1988; Meng et al., 1995). Sequence analysis of nebulin cDNAs reveals that most of the molecule consists of a series of weakly conserved 35-residue modules; most of these modules are organized, in turn, into more highly conserved super repeats, each containing seven modules (Labeit and Kolmerer, 1995; Wang et al., 1996). This organization of residues into modules and super repeats is highly conserved among species (Zhang et al., 1996) and suggests a mode of integration of nebulin into the skeletal muscle thin filament involving interactions with actin as well as regulatory proteins that are present at a 1:7 ratio to actin (Jin and Wang, 1991b; Labeit et al., 1991; Trinick, 1992).

Immunoelectron microscopic studies using site-specific antibodies showed that single nebulin molecules span most of the length of the thin filament (Wang and Wright, 1988; Wright et al., 1993). The molecular masses of nebulin's multiple tissue and developmental specific isoforms range from 600 to 900 kDa and correlate with the length of the thin filaments (Kruger et al., 1991; Labeit et al., 1991). These findings strongly support the hypothesis that nebulin regulates thin filament length and acts as a template for the assembly of thin filaments.

In vitro studies have been hampered by the extremely low solubility of native nebulin and nebulin fragments. Although native nebulin has not been purified to date, recombinant nebulin fragments containing 2–15 modules (Jin and Wang, 1991a,b; Root and Wang, 1994; Wang et al., 1996) as well as synthesized peptides containing a single module (Pfuhl et al., 1994, 1996) have been shown to bind to actin. However, interpretation of these studies is complicated by the finding that the stoichiometry of nebulin modules bound to actin filaments varies severalfold, depending on the particular fragments expressed or synthesized. In addition, some single nebulin modules fail to bind actin at all (Pfuhl et al., 1994, 1996). Finally, cooperativity in binding to actin is observed with only certain nebulin modules (Pfuhl et al., 1996). Therefore, the properties of nebulin binding to actin still remain to be established.

The secondary structures of a highly soluble two-module fragment expressed in *Escherichia coli* (Chen and Wang, 1994) and synthesized single modules soluble in water (Pfuhl et al., 1994, 1996) have been investigated by circular dichroism and nuclear magnetic resonance. All of the nebulin peptides examined show a tendency to fold into transient helical structures around the conserved sequence SDxxYK, and these structures are greatly stabilized by trifluoroetha-

nol and anionic detergents such as sodium dodecyl sulfate (SDS) (Chen and Wang, 1994; Pfuhl et al., 1994, 1996). Furthermore, a progressive increase in both α-helical propensity and actin-binding affinity has been observed for synthetic modules as their corresponding locations in the nebulin molecule become closer to the C-terminus (Pfuhl et al., 1996). It has therefore been suggested that interaction with negatively charged actin residues stabilizes α-helices in the nebulin molecule.

In hopes that their properties would be more representative of the native molecule, we have cloned and expressed much longer fragments of nebulin than previously used. Here we present evidence that interaction with either monomeric or filamentous actin prevents or reverses aggregation of large nebulin fragments. Finally, we have developed methods of incorporating these longer fragments into actin filaments with a stoichiometry resembling that found in vivo.

MATERIALS AND METHODS

Expression of nebulin fragments in *E. coli*

Nebulin fragments were expressed as thioredoxin fusion proteins by using the ThioFusion Expression System (Invitrogen Corporation, San Diego, CA). All molecular cloning techniques were as previously described (Zhang et al., 1996) or as specified in the manufacturer's instructions. Whole inserts of the previously characterized mouse nebulin cDNA clones 7a (2977 bp) and 8c (2000 bp) (Zhang et al., 1996) were gel purified and inserted into a pTrxFus vector at *Bam*HI sites. Bacterial host strain GI724 was transformed with the recombinant plasmids, and plasmids carrying correctly oriented inserts were identified by restriction enzyme digestion. Host cells carrying the expression constructs were first cultured in the induction medium at 30°C until an OD_{550} of 0.5 was reached. Protein expression was induced by adding tryptophan to the culture at a final concentration of 0.1 mg/ml and raising the incubation temperature to 37°C. Then the cells were incubated for an additional 3 h and were harvested by centrifugation at $3500 \times g$ for 10 min. The cell pellets were resuspended in 10 mM imidazole (pH 7.0) and were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Identical conditions were used to express wild-type thioredoxin using a construct supplied by the manufacturer.

Purification of expressed nebulin fragments

The bacterially expressed nebulin fragments 7a and 8c were affinity purified with ThioBond resin (Invitrogen Corporation), and all procedures were based on instructions by the manufacturer, with modifications as specified below. Cell pellets from 3 h postinduced cultures were disrupted in 10 mM imidazole (pH 7.0). After three cycles of sonication followed by freezing and thawing, the cell lysates were centrifuged at $16,000 \times g$ for 20 min. The pellets, which contained the expressed nebulin fusion proteins, were washed and centrifuged two times with the same buffer. The washed pellet was then dissolved in a solubilization buffer containing 6 M urea, 0.5 M NaCl, 1 mM EDTA, and 10 mM imidazole (pH 7.0). After the dissolved material was clarified by centrifugation at $16,000 \times g$ for 20 min, the supernatant was loaded onto a ThioBond affinity column equilibrated in solubilization buffer. After incubation for 1 h, unbound proteins were washed off with the same buffer. Bound nebulin fragments were then eluted with solubilization buffer containing 20 mM β-mercaptoethanol.

Bacterially expressed thioredoxin was similarly purified, except that the supernatant of the cell lysate contained the expressed protein and was loaded onto the ThioBond affinity column.

Solubility studies of nebulin fragments

Because the nebulin fragments were purified in the presence of 6 M urea, we attempted to renature the proteins to native conditions in which biochemical studies could be performed. Two different approaches to renaturation were adopted. The first was to lower the urea concentration gradually by dialyzing the proteins sequentially against 4 M, 2 M, and 1 M urea in a buffer containing 1 M NaCl. The second approach was to first dialyze the protein solution against a basic phosphate buffer (10 mM Na_2HPO_4, pH 12.0) in which smaller expressed nebulin fragments were previously shown to be soluble (Jin and Wang, 1991a). The pH was then lowered by sequential dialysis against 10 mM Na_2HPO_4 (pH 10.0), 10 mM Na_2HPO_4 (pH 9.0), and 20 mM Tris-HCl (pH 8.0). All dialysis was performed at 4°C for 16 h with two changes of buffer. After each step, protein samples were centrifuged at $16,000 \times g$ at 4°C for 20 min, and supernatants were subjected to the next round of dialysis. Supernatants and pellets from each step were analyzed by SDS-PAGE.

Protein assays

Concentrations of pure proteins were determined from absorbance measurements at 280 nm in the presence of 6 M guanidine hydrochloride, using extinction coefficients calculated by the method of Gill and von Hippel (1989). The 7a fusion protein, the 8c fusion protein, thioredoxin, and actin have extinction coefficients of 220,020 M^{-1} cm^{-1}, 147,370 M^{-1} cm^{-1}, 14,180 M^{-1} cm^{-1}, and 44,080 M^{-1} cm^{-1}, respectively, and calculated molecular weights of 129,667, 91,472, 11,806 and 42,006, respectively. The extinction coefficient of actin was calculated by using the amino acid sequence of human actin. Protein concentrations of tissue homogenates were determined with the BioRad Protein Assay kit (BioRad Laboratories, Hercules, CA).

Cosolubilization assays for interaction of nebulin fragments with actin

Purified actin from rabbit skeletal muscle was a generous gift from Dr. James Sellers, and was stored as F-actin in a polymerizing buffer containing 4 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.0), 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), and 3 mM NaN_3. G-actin was prepared by dialysis against a buffer containing 0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$, and 10 mM imidazole (pH 7.0) for 48 h with four buffer changes. The G-actin was clarified by centrifugation at 4°C at $200,000 \times g$ for 20 min in a Beckman Airfuge (Beckman Instruments, Fullerton, CA).

Interaction of nebulin fragments with G-actin was demonstrated by a cosolubilization assay. Purified nebulin fragments 7a and 8c were dialyzed against the 10 mM Na_2HPO_4 (pH 12) buffer, as described in the renaturation section. Varying amounts of G-actin were added to the nebulin fragments in pH 12 phosphate buffer, and the volume was adjusted with phosphate buffer so that the nebulin fragments were at a concentration of 1 μM. The mixture was dialyzed at 4°C against the G-actin buffer for 16 h with two buffer changes, followed by centrifugation at $16,000 \times g$ for 20 min. In each case, the pellets were redissolved overnight in the 10 mM Na_2HPO_4 (pH 12) buffer, using the same volumes used for the initial nebulin and actin mixtures. Equal amounts of supernatant and pellet were analyzed by SDS-PAGE for each particular G-actin concentration.

A cosolubilization assay was also used to assay the interaction of nebulin fragments with F-actin. Purified nebulin fragments were dialyzed against an actin-polymerizing buffer containing 4 mM MOPS (pH 7.0), 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 100 mM NaCl, and 1 mM ATP. We found that the suspended precipitates could be accurately pipetted with slick tips manufactured by PGC (PGC Scientifics Corp., Gaithersburg, MD). Reaction mixtures were assembled that contained 4 μM actin and varying concentrations of aggregated nebulin fragments 7a or 8c in a final volume of 15 μl of actin-polymerizing buffer. The mixtures were incubated at 4°C overnight and then centrifuged at $5220 \times g$ for 10 min in a benchtop

centrifuge. Pellets were solubilized in gel sample buffer, and the supernatants and pellets were analyzed by SDS-PAGE.

In experiments to control for nonspecific binding, BSA was used to replace either F-actin or G-actin in the cosolubilization assays. In addition, binding of thioredoxin to actin filaments was assayed under the same conditions used to assay nebulin solubilization by actin filaments. In these experiments, bound thioredoxin was cosedimented with the actin filaments by centrifugation at $200,000 \times g$ for 20 min, and the pellets and supernatants were analyzed by SDS-PAGE. No specific binding of thioredoxin to F-actin was observed (data not shown).

Fluorescence microscopy

Nebulin fragments 7a and 8c were reacted with actin filaments as described above. After centrifugation at $5220 \times g$ for 10 min, supernatants containing the nebulin-F-actin complexes were labeled with either a monoclonal anti-nebulin antibody (clone NB2 from Sigma Chemical Company, St. Louis, MO) or with rhodamine phalloidin (Molecular Probes, Eugene, OR). The monoclonal anti-nebulin antibody was added to each sample at a dilution of 1:100 and incubated overnight at 4°C. Tetramethylrhodamine goat anti-mouse IgG (Molecular Probes) was then added at a 1:100 dilution, and the mixture was incubated overnight at 4°C. Pelleted 7a and 8c aggregates were resuspended in F-actin buffer and similarly stained with the anti-nebulin antibody for comparison. Parallel samples of actin filaments and actin-nebulin complexes were stained overnight with equimolar concentrations of rhodamine phalloidin at 4°C.

Nitrocellulose (superclean grade; Ernest F. Fullam, Inc., Latham, NY)-coated coverslips and flow cells were prepared as previously described (Sellers et al., 1993). F-actin and actin-nebulin complexes were diluted to a final actin concentration of 1 μM immediately before being introduced to the flow cells; the final buffer also contained 10 mM DTT and oxygen-scavenging agents (2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase, and 0.02 mg/ml catalase, all purchased from Sigma Chemical Company) to prevent photobleaching (Sellers et al., 1993). The samples were allowed to incubate in the flow cells for 10 min before viewing.

Samples were viewed with a Zeiss Axiovert 135 microscope equipped for incident-light fluorescence, using a 100× plan-neofluar oil immersion objective with a numerical aperture of 1.30 (Carl Zeiss, Thornwwod, NY). Images were digitized with a CCD camera attached to a Hamamatsu Argus-20 image processor (Hamamatsu Photonics K. K., Hamamatsu City, Japan) and transferred to a personal computer for analysis. Filament lengths and particle sizes were quantitated on a Macintosh computer using the public domain National Institutes of Health Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Electron microscopy

Nebulin fragments 7a and 8c were reacted with actin filaments as described above. After centrifugation at $5220 \times g$ for 10 min, supernatants containing the nebulin-F-actin complexes were applied to carbon-coated 400 mesh copper grids. The samples were negatively stained with 1% aqueous uranyl acetate. They were viewed and photographed with a Philips model CM100 electron microscope.

Preparation and analysis of myofibrillar proteins from mouse skeletal muscle

The preparation procedure for myofibrils was based on the method of Trinick et al. (1984). Skeletal muscle was excised from the hind legs and backs of mice. Using an Omni 2000 homogenizer (Omni International, Gainesville, VA), we homogenized the muscle in 10 volumes of ice-cold rigor buffer (100 mM KCl, 2 mM MgCl$_2$, 1 mM EDTA, 0.5 mM DTT, 10 mM Imidazole-HCl, pH 7.0), with the addition of 10 μg/μl phenylmethylsulfonyl fluoride and 10 μg/μl leupeptin to minimize possible protein degradation. The homogenate was centrifuged at $2000 \times g$ for 10 min. The

myofibril pellet was resuspended, washed, and centrifuged twice more with the same buffer. The protein concentration of the final suspension of myofibrils was estimated using the Bio-Rad Protein Assay Kit (Bio-Rad) before analysis by SDS-PAGE.

Varying amounts of 8c, actin, and myofibrils were loaded on the same gel. For each of three myofibril preparations, linear relations were obtained between peak area and protein loading for pure 8c and actin, as well as for the myofibrillar nebulin and actin bands. An accurate estimate of myofibrillar nebulin and actin content was obtained by dividing the slope of the regression lines relating these band intensities to myofibrillar protein loading by the slopes obtained for the corresponding pure proteins. This analysis assumes that the pure fusion proteins and muscle nebulin bind the same amount of stain per microgram. Because 97% of the nebulin sequence consists of repeating modules of the type found in our expressed fragments (Labeit and Kolmerer, 1995), this assumption is reasonable, provided that the thioredoxin moiety does not bind a disproportionate amount of stain. Control experiments showed that the cloned nebulin fragments and actin bound roughly equivalent amounts of dye per microgram of protein, and that this amount was more than twice that bound by pure thioredoxin. The myofibrillar ratio of nebulin modules per actin monomer was calculated by assuming that the entire nebulin molecule is composed of modules with an average molecular weight of 4179. This value is derived from the published full-length sequence of human nebulin, which shows that 97% of the nebulin molecule is composed of repeating modules (Labeit and Kolmerer, 1995).

Protein gel electrophoresis

SDS-PAGE was performed by standard procedures, and proteins were visualized with Coomassie brilliant blue R-250 (Bio-Rad). In general, precast mini-gels with a 4–20% gradient of polyacrylamide were run in BioRad's Mini-Protean II electrophoresis chambers (BioRad), using the buffer system of Laemmli (1970). For the purpose of resolving large myofibrillar proteins, gels containing a 2–20% gradient of polyacrylamide were prepared with the Fairbanks' continuous buffer system (Fairbanks et al., 1971). It should be noted that in sample preparation, the myofibrillar proteins containing titin and nebulin were boiled for no more than 2 min to avoid degradation of these large proteins.

Immunoblots

Expressed nebulin fusion proteins were initially detected on immunoblots by using a monoclonal anti-thioredoxin antibody (Invitrogen Corp.). Proteins separated on Laemmli gels were transferred onto nitrocellulose membranes by the method of Towbin et al. (1979), using BioRad's Mini-Protean II transfer chambers (BioRad) set at 100 V for 1.25 h. Membranes were blocked with phosphate-buffered saline (PBS) containing 0.1% Tween (PBS-T) and 5% non-fat dry milk for 1 h, and then extensively washed with PBS-T. Primary and secondary antibodies (horseradish peroxidase conjugated antibody from sheep against mouse immunoglobulins) were diluted in PBS-T to final concentrations of 1:5,000 and 1:10,000, respectively. The incubations with antibodies were carried out at room temperature for 1 h each and were followed by extensive washing in PBS-T. The ECL Western Blot System was used for detection of bound antibody (Amersham Corporation, Arlington Heights, IL).

The reactivity of an anti-nebulin monoclonal antibody was tested with the expressed nebulin fragments, as well as with total proteins from mouse skeletal and cardiac muscle. Mouse tissues were dissected, frozen in liquid nitrogen, and stored at -80°C until use. Tissues were homogenized in $\sim$12 volumes of a buffer composed of 20 mM Tris, 2 mM EDTA, 20 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride, pH 8.0, using an Omni 2000 homogenizer (Omni International). The homogenates were diluted 1:1 with 2% SDS, 20% glycerol, 140 mM β-mercaptoethanol, and 0.05% bromophenol blue. Samples were boiled for 1–2 min, and the proteins were separated on Fairbanks gels. The proteins were transblotted onto PVDF (polyvinylidene difluoride) paper in a transfer buffer contain-

ing 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol, 0.1% SDS, and 10 mM β-mercaptoethanol. Transfer was performed at room temperature overnight in a Hoefer transblotting unit (Hoefer Scientific Instruments, San Francisco, CA) at 300 mA. The PVDF blots were blocked, probed, and detected as described above, except that PBS-T contained 0.2% Tween-20, both the blocking solution and antibody solutions contained 10% nonfat dry milk, and the primary antibody was monoclonal anti-nebulin (clone NB2) from Sigma Chemical Company, used at a final dilution of 1:400.

Gel densitometry analysis

Photographs of gels were digitized with a flatbed scanner controlled by a Macintosh computer. Integrated densities of protein bands were obtained by using National Institutes of Health Image. The linearity of peak areas with the amount of protein loaded was verified for all experiments.

RESULTS

Expression of mouse skeletal muscle cDNA clones encoding nebulin

The sequences of the mouse nebulin cDNA clones 8c and 7a can be assembled into one contiguous sequence, as previously reported (Zhang et al., 1996) (Genbank accession number U58108). Clone 8c corresponds to the first 1977 nucleotides of this sequence, and clone 7a corresponds to nucleotides 1123–4076. In the present study, these two overlapping cDNA clones were cloned into the pTrxFus expression vector. The 7a and 8c inserts encode mouse nebulin fragments consisting of four full super repeats (28 modules) and 2.7 super repeats (19 modules), respectively, derived from the central region of the molecule. These inserts were fused in frame with residues encoding thioredoxin, as well as with residues within the multiple cloning sites in the vector.

Protein expression was induced upon the addition of tryptophan to the induction medium. Total cell protein from preinduced and 3-h postinduced samples was analyzed by SDS-PAGE (Fig. 1, *left hand panels, lanes 1 and 2*). In the postinduced protein samples, new protein bands can be seen, with calibrated molecular weights matching the calculated sizes of the fusion proteins. Fig. 1 also shows that the overexpressed proteins are detected by an anti-thioredoxin antibody, positively identifying them as the expected products (*right hand panels, lanes 2*). Some breakdown products were also detected with the anti-thioredoxin antibody. It was evident that cells expressed fusion proteins specifically under tryptophan induction, because there was no expression of fusion proteins in the uninduced samples (Fig. 1, *lanes 1*), indicating tight regulation by the P_L promoter. The fusion proteins were estimated by gel densi-
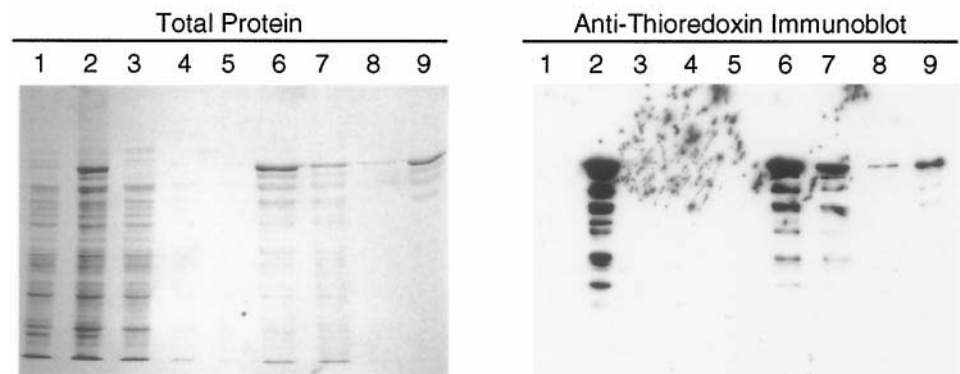

FIGURE 1 Coomassie blue-stained gels (*left-hand panels*) and corresponding immunoblots (*right-hand panels*) illustrating the protocol for purifying 7a (*A*) and 8c (*B*). *Lanes 1*: Total lysate from uninduced cell culture; *lanes 2*: 3-h postinduced cell culture; *lanes 3*: 10 mM imidazole (pH 7.0)-soluble proteins; *lanes 4 and 5*: supernatants from two more wash steps with the 10 mM imidazole (pH 7.0) buffer; *lanes 6*: pelleted material dissolved in solubilization buffer containing 6 M urea; *lanes 7*: flow-through fraction from the column; *lanes 8*: unbound proteins washed off the column with urea buffer; *lanes 9*: purified protein eluted from the column.
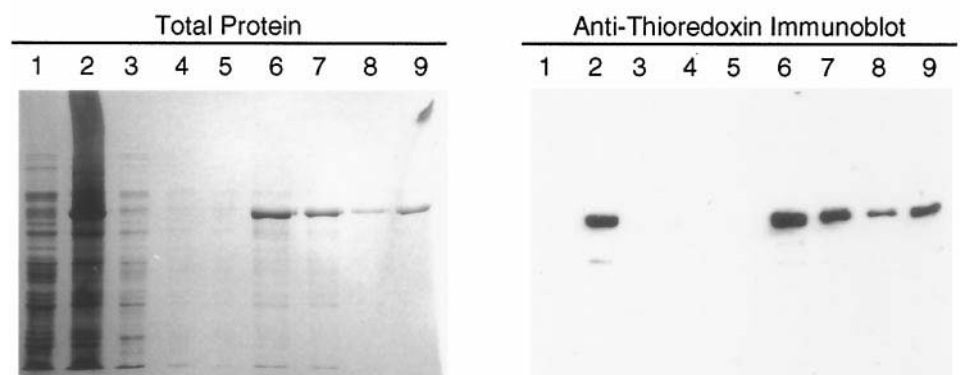
tometry to comprise as much as 40% of the total cell protein, suggesting a high degree of stability of mouse nebulin fragments in the host cells, as reported previously for smaller fragments of human nebulin (Jin and Wang, 1991a, 1991b).

Purification of expressed nebulin fragments

Taking advantage of the thioredoxin moiety within the nebulin fusion proteins, we developed a simple procedure utilizing a ThioBond affinity column to purify the expressed nebulin fragments. The ThioBond resin was particularly useful because intact fusion proteins bound to the resin, while degradation products that do not contain the thioredoxin domain of the fusion proteins were not retained by the column. Protein samples from each step of the purification procedure were analyzed by gel electrophoresis and immunoblot with the anti-thioredoxin antibody (Fig. 1). Disrupting the cells in a buffer containing 10 mM imidazole (pH 7.0) solubilized most of the *E. coli* proteins, thereby enriching the pelleted fractions with the expressed nebulin fragments. The pelleted material was dissolved in a high salt buffer containing 6 M urea and loaded onto the ThioBond affinity column. Flow-through and wash steps removed smaller peptides, presumably degradation products of 7a and 8c. The final protein preparations eluted from the column in the presence of urea were highly purified nebulin fusion proteins (Fig. 1, *lanes 9*). An average yield of 25 mg of fusion proteins 7a or 8c per liter of culture could be readily obtained within several hours by this method.

A monoclonal anti-nebulin antibody detected both the affinity-purified 7a and 8c fusion proteins on immunoblots, as well as trace amounts of smaller bands that appeared to be degradation products of the fusion proteins (Fig. 2). The anti-nebulin antibody also specifically detected nebulin in immunoblots of mouse skeletal muscle proteins, but failed to detect any bands in mouse cardiac muscle. These results positively identify the purified thioredoxin fusion proteins as the expected nebulin fragments.

Solubility of purified nebulin fragments

Previous studies have shown that nebulin and nebulin fragments are extremely insoluble under native conditions (Wang, 1985; Jin and Wang, 1991b). One motivation for expressing nebulin fragments as thioredoxin fusion proteins is that this system often confers solubility on otherwise insoluble proteins (LaVallie et al., 1993). Unfortunately, this was not the case in the present study. When the urea concentration was gradually lowered by sequential dialysis, both 7a and 8c remained soluble in the presence of 4 M and 2 M urea, but formed insoluble aggregates at urea concentrations of 1 M or less. Like full-length nebulin, nebulin fragments 7a and 8c are very basic, having predicted isoelectric points well over pH 9.0. Dialysis to pH 12 completely solubilized 7a and 8c. However, when nebulin frag-
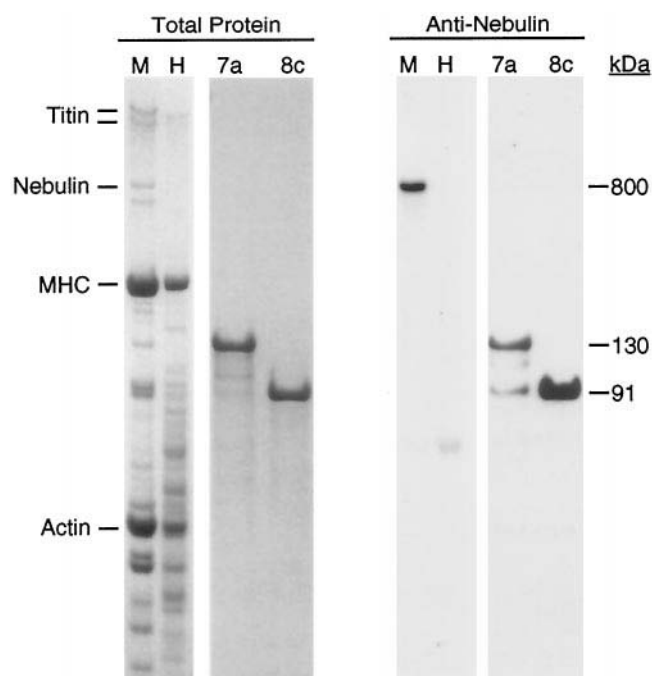

FIGURE 2 Coomassie blue-stained gel (*left-hand panel*) and corresponding immunoblot (*right-hand panel*) illustrating the detection of expressed 7a and 8c with an anti-nebulin antibody. Lanes were loaded with total proteins from mouse skeletal muscle (M) and heart (H), as well as purified 7a and 8c. The antibody detects recombinant nebulin fragments as well as skeletal muscle nebulin. No proteins are detected in the heart, which does not express nebulin. MHC, Myosin heavy chain.

ments were then sequentially dialyzed to lower pH values, solubility decreased. Fragment 7a was completely insoluble at pH 9.0 and below, whereas 8c was completely insoluble at pH 8.0 and below. Although Root and Wang (1994) previously reported that calcium significantly enhances the solubility of a nebulin fragment composed of a single super repeat, we found no difference in the solubilities of 7a or 8c when 1 mM $CaCl_2$ was added to all dialysis buffers. In summary, our studies showed that purified nebulin fragments 8c and 7a are almost completely insoluble at neutral pH in the absence of denaturants (Fig. 5, *A* and *E*).

Interaction between nebulin fragments and monomeric actin

We hypothesized that interaction with actin might prevent nebulin fragments from forming insoluble aggregates. To test this idea, we mixed constant amounts of purified 8c and 7a with varying amounts of G-actin at pH 12, and then dialyzed the mixtures to neutral pH. We then assayed the proteins for solubility by centrifugation at $16,000 \times g$ and analyzed the pellets and supernatants by gel electrophoresis. We observed that the monomeric actin conferred apparent solubility on the nebulin fragments in a concentration-dependent manner (Fig. 3, *top panels*). Fig. 4 shows the dependence of 7a and 8c solubility on G-actin concentration. The data show that micromolar concentrations of G-

FIGURE 3 Solubilization of expressed nebulin fragments 8c (*A*) and 7a (*B*) by G-actin. Reactants were mixed at pH 12 to yield mixtures containing 1 μM 8c or 7a and varying concentrations of actin, as indicated. After dialysis to G-actin buffer at pH 7.0, the mixtures were centrifuged at 16,000 $\times$ *g*, and the pellets (P) and supernatants (S) were analyzed by gel electrophoresis. The presence of G-actin caused a shift of 8c and 7a from the pellet to the supernatant fractions (*top panels*). When BSA was substituted for G-actin, the nebulin fragments were not solubilized (*bottom panels*), illustrating the specificity of the interaction between G-actin and the nebulin fragments 7a and 8c. Note that an impurity in the BSA preparation migrates just above 7a (see Fig. 5 *C*).
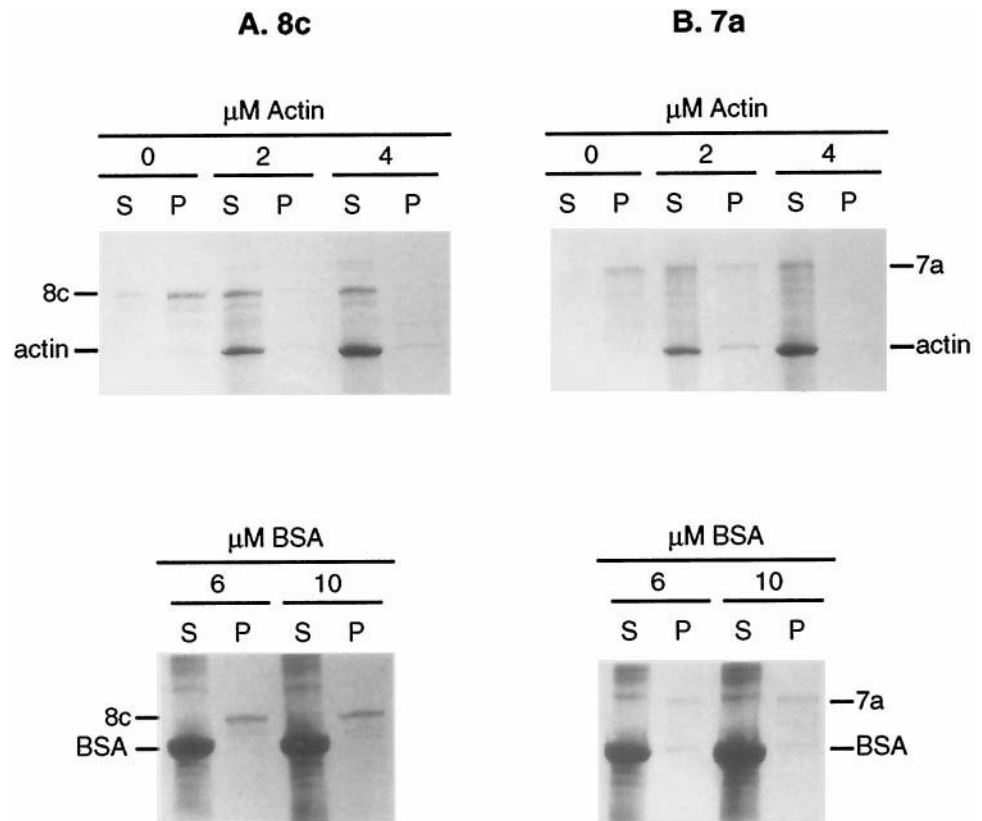
actin can completely solubilize nebulin fragments containing two to four super repeats. This effect could not be reproduced by substituting BSA for actin; even at higher concentrations of BSA, the nebulin fragments 8c and 7a remained insoluble when dialyzed to neutral pH (Fig. 3,

bottom panels). Because BSA is in the same size range as monomeric actin and has a similar isoelectric point, these results show that the effect of actin on nebulin solubility is not due simply to nonspecific interactions between oppositely charged proteins.
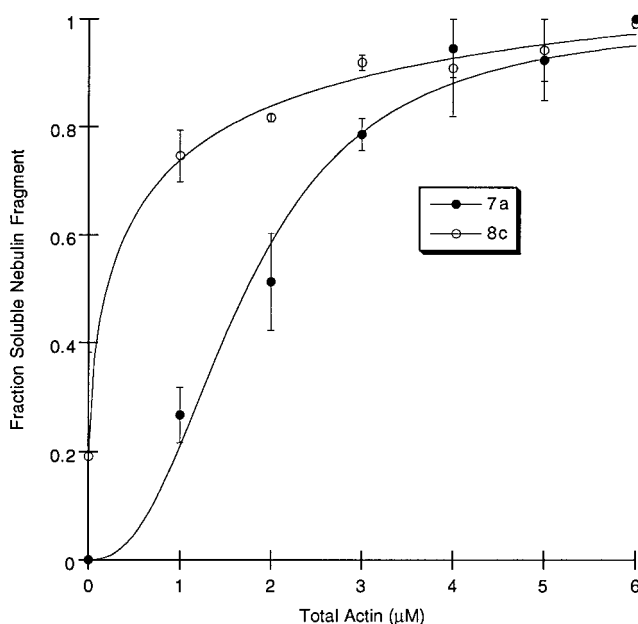
Interaction of nebulin fragments with actin filaments

The extremely low solubility of 8c and 7a precluded measuring their interaction with actin filaments by the widely used cosedimentation assay for actin binding. As an alternative approach, we used the ability of actin to solubilize nebulin fragments as a way to assay the association of 7a and 8c with actin filaments.

When varying amounts of insoluble 7a or 8c were mixed with F-actin, we found that small amounts of the nebulin fragments remained in the supernatant with the bulk of the actin filaments after centrifugation at low speed (Fig. 5, *B* and *F*). When actin was omitted or replaced by BSA, no measurable 7a or 8c remained in the supernatants (Fig. 5, *A*, *E*, *C*, and *G*). Furthermore, we found that 1 μM 7a or 8c could be almost completely transferred to the supernatant fraction by the addition of a large excess of actin filaments (Fig. 5, *D* and *H*). These results suggest that the nebulin fragments found in the supernatant fraction were incorporated into the actin filaments.

We quantitated the amount of 7a and 8c in the pellet and supernatant fractions by densitometry. The linearity of the

FIGURE 4   Concentration dependence of 8c and 7a solubilization by G-actin. Each point is the mean $\pm$ SEM of two experiments.
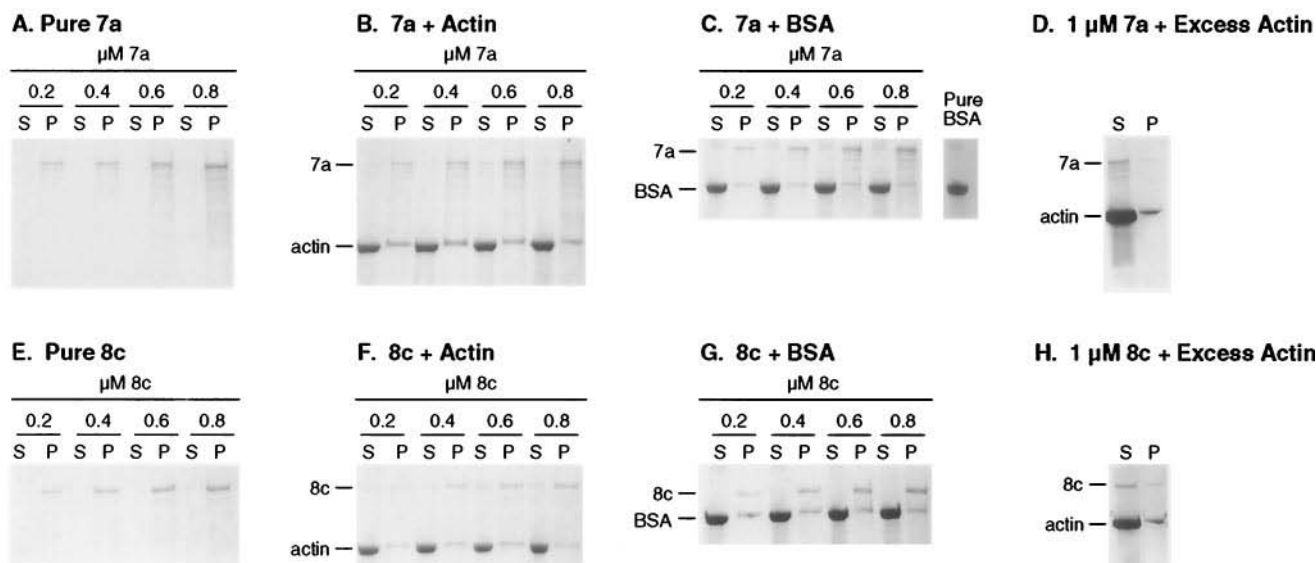
FIGURE 5 Incorporation of aggregated nebulin fragments 7a and 8c into preformed actin filaments. Reaction mixtures were assembled in an actin-polymerizing buffer to yield the indicated concentrations of either pure 7a and 8c (*A* and *E*), or 7a and 8c in the presence of 4 μM actin (*B* and *F*) or BSA (*C* and *G*). After incubating overnight, the mixtures were centrifuged at 5220 × *g*, and the supernatants (S) and pellets (P) were analyzed by gel electrophoresis. No 7a or 8c is detected in the supernatant fractions when incubated alone (*A* and *E*) or in the presence of BSA (*C* and *G*). However, small amounts of 7a or 8c are found in the supernatant after incubation with F-actin (*B* and *F*). Under these conditions, 10–15% of the actin was found in the pellet; this amount did not vary as the 7a or 8c concentration was varied from 0 to 1.6 μM (data not shown). Note that the BSA preparation contains an impurity that migrates just above 7a (*C*, pure BSA). In a reaction mixture containing 1 μM 7a and 30 μM F-actin (*D*) or 1 μM 8c and 25 μM F-actin (*H*), almost all of the nebulin fragments remain in the supernatant with the actin filaments.

measurments was verified for each gel analyzed, as illustrated by the typical example shown in Fig. 6. Fig. 6 shows that over the entire range of concentrations used in these experiments, the sum of the peak areas measured in the pellet and supernatant fractions was linearly related to the total amount of 7a and 8c in the reaction mixtures.

Fig. 7 shows the nebulin fragment-to-actin stoichiometries found in the supernatants plotted as a function of
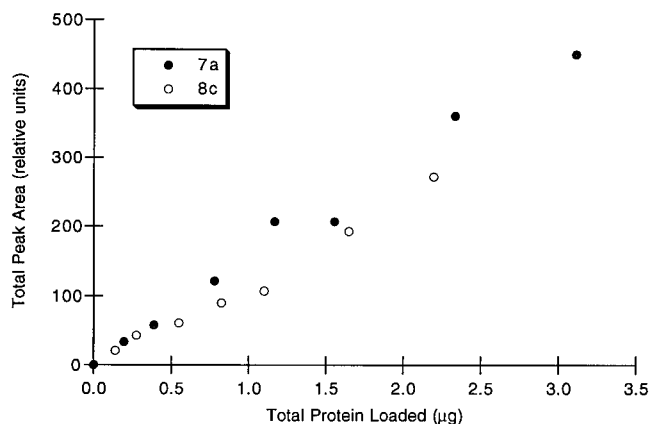

FIGURE 6　Total peak area of the 7a or 8c bands versus the total amount of 7a or 8c in the reaction mixture. Each point represents the sum of the appropriate peak areas measured in the pellet and supernatant fractions for reaction mixtures such as those shown in Fig. 5. The data show that 7a and 8c staining is quantitative and linear over the range of concentrations studied. Linearity of the signal was verified for each gel analyzed in this study.

pelleted 7a and 8c. These binding curves were fit by the Hill equation (Hill, 1910), yielding apparent dissociation constants in the submicromolar range and cooperativity parameters that were not significantly different from 1 (Table 1). In addition, we calculated that 7a binds to actin filaments with a stoichiometry equivalent to one nebulin module per actin monomer, whereas 8c is incorporated to a level of only one nebulin module per two actin monomers (Table 1).

Morphology of the actin-nebulin complexes

To verify that the 7a and 8c in the supernatant fractions were incorporated into actin filaments, we stained aliquots of the putative complexes with either an anti-nebulin antibody or with phalloidin. For comparison, we also examined the pelleted nebulin aggregates after staining with the anti-nebulin antibody. The insoluble 7a and 8c pellets appeared as punctate aggregates when stained with the anti-nebulin antibody (Fig. 8, *A* and *B*). In contrast, the actin-nebulin complexes appeared filamentous (Fig. 8, *C* and *D*). Actin filaments alone were not stained with the anti-nebulin antibody (Fig. 8 *E*). When stained with phalloidin, complexes of 7a or 8c with actin appeared identical to actin filaments alone (Fig. 8, *F–H*). The punctate nebulin aggregates averaged 0.9 μm in diameter, whereas the nebulin-actin filaments averaged 2.4 μm in length when stained with the anti-nebulin antibody (Fig. 9, *open bars*). Actin filaments and actin-nebulin complexes stained with phalloidin were somewhat longer, their mean lengths ranging from 3.1 to 3.4
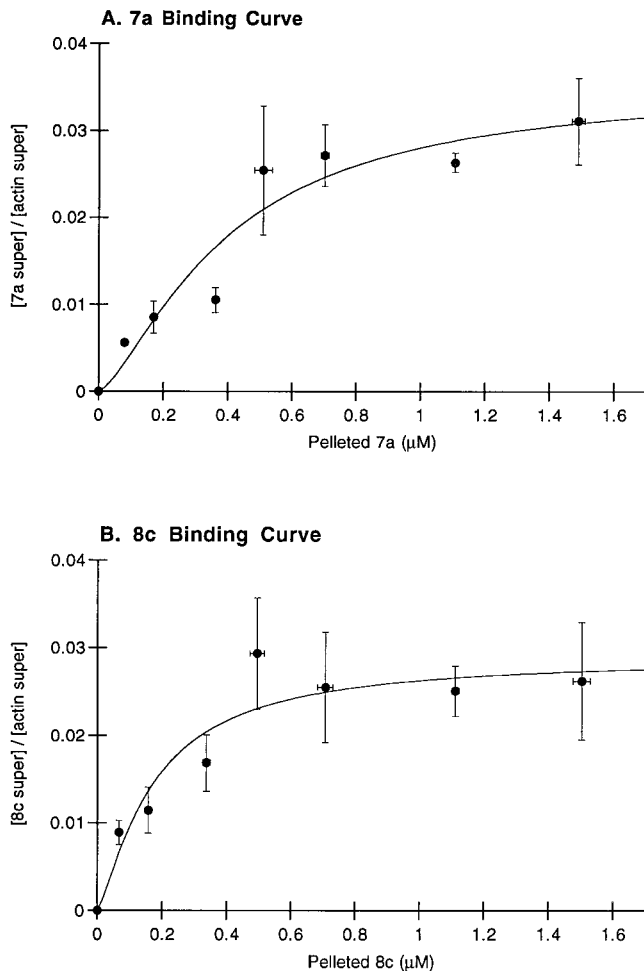
A. 7a Binding Curve

B. 8c Binding Curve

FIGURE 7   Apparent binding isotherms for incorporation of 7a (*A*) and 8c (*B*) into actin filaments. Each point represents the mean $\pm$ SEM of four experiments. Solid curves represent the best fit of the data to the Hill equation (Hill, 1910).

μm (Fig. 9, *hatched bars*). These results demonstrate that nebulin fragments in the pelleted fraction form large, insoluble aggregates, whereas nebulin fragments transferred to the supernatant fractions by interaction with actin filaments are organized into long, filamentous structures.

We examined the putative nebulin-actin complexes in the electron microscope after negative staining. Fig. 10 shows typical actin filaments, as well as actin-7a and actin-8c complexes. At this resolution, the nebulin-actin complexes are indistinguishable from pure actin filaments. All of the filaments observed in these samples had morphologies typical of actin filaments. These results indicate that the filamentous structures observed by immunofluorescence with the anti-nebulin antibody were a result of the incorporation of 7a and 8c into the preformed actin filaments.

Stoichiometry of nebulin and actin in myofibrils

We used purified 8c and actin to calibrate the staining intensities of nebulin and actin, respectively, after separa-

tion of myofibrillar proteins by gel electrophoresis. We found that myofibrils from mouse skeletal muscle contain 20.8 $\pm$ 6.2% actin by weight, in agreement with previous estimates for rabbit myofibrils (Yates and Greaser, 1983). Mouse myofibrils contain 2.48 $\pm$ 0.80% nebulin by weight. By using the average molecular weight of the 185 nebulin modules found in human nebulin (Labeit and Kolmerer, 1995), we could calculate directly the molar ratio of nebulin modules to actin monomers in the mouse myofibrils. This molar ratio is 1.19 $\pm$ 0.10 (mean $\pm$ SEM of three independent experiments).

DISCUSSION

Investigation of nebulin function in vitro has been hampered by the extremely low solubility of this large molecule under physiological conditions. This difficulty also arises with recombinant fragments of nebulin, and has limited previous studies to fragments containing less than two super repeats. In this study, we have used bacterially expressed recombinant fragments of nebulin containing up to four super repeats to explore the effects of actin binding on nebulin solubility and aggregation at neutral pH. These experiments demonstrated that previously insoluble nebulin fragments can become soluble at neutral pH via interaction with actin, making it possible to study large nebulin fragments, formerly stable only under denaturing conditions, in a native buffer environment.

Initial experiments showed that micromolar concentrations of G-actin could keep nebulin super repeats soluble when dialyzed to neutral pH. When suspensions of aggregated nebulin fragments were incubated with preformed actin filaments, the long nebulin fragments were incorporated into the filaments in a concentration-dependent manner. The filament lengths measured after labeling of the actin-nebulin complexes with an anti-nebulin antibody were somewhat shorter than after labeling with phalloidin. Because phalloidin stabilizes actin filaments by inhibiting subunit dissociation at both ends (Sampath and Pollard, 1991), this difference in filament length is likely due to partial depolymerization of the samples without phalloidin upon dilution from 4 μM to 1 μM actin for observation in the fluorescent microscope. Finally, although a previous report suggests that phalloidin may cause nebulin to dissociate from actin filaments (Ao and Lehrer, 1995), the key point of the fluorescence studies is that the anti-nebulin antibody detects structures morphologically similar to actin filaments, as detected by phalloidin. Examination of these samples by electron microscopy verified that the only filamentous structures present were actin filaments.

Taken together, these findings imply that the apparent insolubility of nebulin arises from interaction of nebulin molecules to form multimolecular aggregates; although aggregated, the nebulin molecules still display binding sites that can interact with actin filaments, which incorporate the nebulin fragments and dissociate them from the aggregated

TABLE 1 Binding parameters

	Nebulin fragments		Binding parameters		
Reference	Fragment name	Fragment length (modules)	Stoichiometry (modules/actin)	K_d (μM)	Hill coefficient
This report	Myofibrillar	~185	1.19 ± 0.10		
This report	7a	28	1.00 ± 0.27	0.40 ± 0.20	1.42 ± 0.67
This report	8c	19	0.55 ± 0.10	0.17 ± 0.08	1.29 ± 0.66
Root and Wang (1994)	NA3	8	12.8 ± 0.8	0.9 ± 0.2	
Root and Wang (1994)	NA4	7	6.65 ± 0.42	0.13 ± 0.05	
Pfuhl et al. (1996)	S6R1	1	no binding		
Pfuhl et al. (1996)	S6R2	1	2.74 ± 0.21	240 ± 100	2.21 ± 0.92
Pfuhl et al. (1996)	S6R7	1	1.17 ± 0.05	430 ± 34	2.50 ± 0.07
Pfuhl et al. (1996)	S20R1	1	0.42 ± 0.11	45 ± 11	1.36 ± 0.30

nebulin. This property formed the basis of a novel cosolubilization assay for nebulin incorporation into actin filaments. We found that 7a and 8c bound to actin filaments with dissociation constants in the submicromolar range (Table 1). Interestingly, the stoichiometry of binding on a module-per-actin basis differed between the two fragments: 8c incorporation into actin filaments saturated at a level equivalent to 0.55 modules per actin monomer, whereas 7a

incorporation saturated at a level of 1.00 module per actin monomer.

Table 1 compares the binding parameters we obtained for 7a and 8c incorporation into actin filaments with parameters obtained by others in cosedimentation assays, using cloned or synthesized nebulin fragments of varying lengths. The large variation in the binding stoichiometry of nebulin fragments to actin filaments illustrates the difficulty of forming a nebulin-actin cofilament in vitro that resembles the complex formed in vivo. Pfuhl et al. (1996) found that although one particular nebulin module did not bind to actin filaments, three other single nebulin modules bound to actin filaments with dissociation constants ranging from 45 to 240 μM. The stoichiometry of binding for these single modules ranged from 0.42 to 2.74 modules per actin monomer; only one synthetic module bound to actin at a 1:1 molar ratio. Root and Wang (1994) studied cloned nebulin fragments containing seven or eight modules (i.e., one complete super repeat). These constructs bound to actin filaments with dissociation constants in the submicromolar range, similar to 7a and 8c. However, the single super repeats bound to actin filaments with a stoichiometry of one
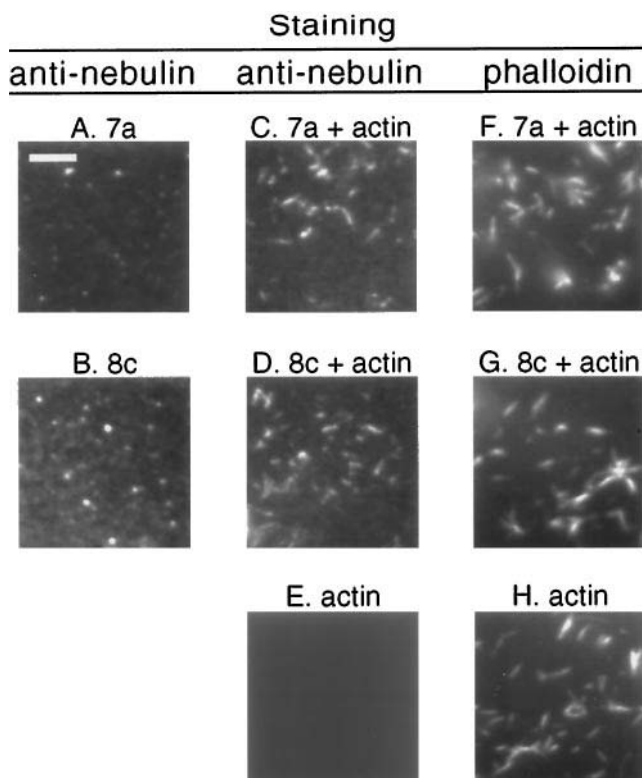

FIGURE 8 Fluorescent images of samples stained with either anti-nebulin (A–E) or phalloidin (F–H). Reaction mixtures containing 4 μM actin filaments and 2 μM 7a or 8c were used. The insoluble pellets (A and B) as well as the supernatant fractions (C and D) were stained with anti-nebulin. Actin filaments incubated without 7a or 8c were not stained by the antibody (E). For comparison, the actin filaments in the supernatant fractions were also directly stained with rhodamine-phalloidin (F–H). Calibration bar in A: 10 μm.
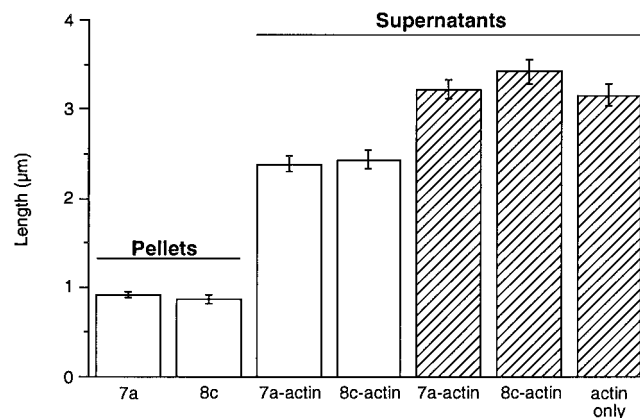

FIGURE 9 Mean dimensions of the fluorescent particles detected by the anti-nebulin antibody (□) and phalloidin (▨). The mean particle diameter is shown for insoluble 7a and 8c pellet fractions, and the mean filament length is shown for the supernatant fractions containing actin. Error bars represent the standard error of the mean.
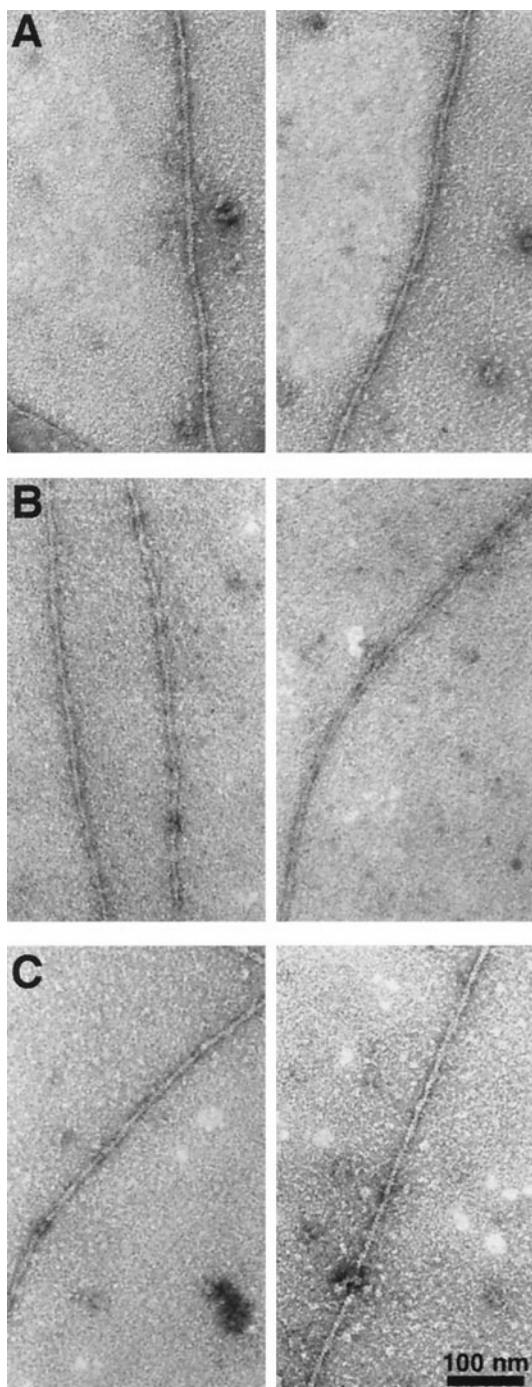
FIGURE 10   Electron micrographs of negatively stained actin filaments (*A*) and actin filaments containing 7a (*B*) and 8c (*C*).

into actin filaments with a stoichiometry near that found in myofibrils. Our findings suggest that it may be possible to reconstitute the thin filament, complete with nebulin and regulatory proteins, in vitro. The methods we have developed should prove useful in evaluating the fine structure of the actin-nebulin cofilaments, as well as whether nebulin plays a physiological role in the regulation of muscle contraction.

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to two super repeats per actin monomer; this is equivalent to 6.6–12.8 nebulin modules per actin monomer (Table 1). Complexed in this manner, the single super repeats have an inhibitory effect on actomyosin ATPase activity and sliding velocity (Root and Wang, 1994). However, the abnormal stoichiometry of these actin-nebulin complexes raises the question of the physiological significance of this finding.

In contast, in this study we have succeeded in incorporating nebulin fragments containing several super repeats

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