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Nebulin as a giant actin-binding template protein in skeletal muscle sarcomere

Interaction of actin and cloned human nebulin fragments

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Nebulin is a family of giant sarcomere matrix proteins of 600–900 kDa in most vertebrate skeletal muscles. Recent sequence analysis suggests that human nebulin is mainly composed of a large number (> 200) of conserved repeats of ~35 residues. Two cloned nebulin fragments, consisting of 6 and 8 of the repeats, have been expressed in *E. coli* using the pET3d vector. Both F-actin cosedimentation and solid-phase binding assays demonstrated a specific binding of these nebulin fragments to actin. This finding suggests that nebulin is a giant protein which binds actin at multiple sites in a template-manner. The presence of an actin-binding template protein in the skeletal muscle sarcomere may have significant implications in the assembly and function of the contractile apparatus.

Nebulin; Actin-binding protein; Muscle thin filament; cDNA expression in *E. coli*; ELISA

1. INTRODUCTION

Nebulin, a family of giant sarcomere matrix proteins of 600–900 kDa in most vertebrate skeletal muscles [1–4], has been proposed to constitute a set of inextensive longitudinal filaments which are attached to the Z line and are coextensive with the actin filaments [2]. The potential role of nebulin as a template protein which interacts with actin and regulates thin filament length has gained some experimental support [2–6]. Molecular cloning and sequencing studies [7–10] on human nebulin cDNA have revealed unique repeating motifs in the protein sequence at two levels: a very well conserved ~35-residue module repeated at least 200 times throughout most of the molecule and a higher order repeat of ~240 residues consisting of seven ~35-residue modules [7]. We hypothesize that the long chain of these ~35-residue modules may form a structural scaffold or template which matches the three dimensional contour and charge profiles of actin in thin filament and that the ~240-residue repeats may correspond to the dimension of the seven-actin thin filament periodicity. To evaluate this hypothesis, we have expressed several cloned human nebulin fragments each containing 6–8 of the ~35-residue modules in *E. coli* and demonstrated their specific binding to muscle actin.

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2. EXPERIMENTAL

2.1. *Engineering of the expression plasmids*

Two contiguous *Nco*I restriction fragments, HNd4nB5 (674 bp) and HNd4nA3 (809 bp), were prepared from a 4.5 kb cDNA clone (HNd4) encoding human skeletal muscle nebulin [7–9]. The two cDNA fragments code for protein fragments NB5 (224 residues) and NA3 (269 residues), and both proteins would start and end with methionine residues corresponding to the *Nco*I-cutting sequences. In order to express non-fusion human nebulin fragments by the pET3d translational expression plasmid vector [11], a synthetic *Nco*I–*Bam*HI adaptor which contains translation termination codons in all three reading frames [12] was introduced at the downstream-end of the cDNA insert. The *Nco*I-cut cDNA fragments were first ligated to the *Nco*I–*Bam*HI-cut vector DNA by their common *Nco*I generated ends and then the translation termination adaptor was added to join the remaining free *Nco*I-cut end of cDNA insert to the *Bam*HI-cut end of vector DNA. After transformation of competent JM109 *E. coli* cells, ampicillin resistant colonies were screened for the insert-bearing recombinant plasmids with corresponding size shifts. The expression plasmid constructs were further examined by restriction mapping and DNA sequencing to select the correct insert orientation and verify the translation reading frame and termination site.

2.2. *Expression of human nebulin fragments in* E. coli

After transformation of the two expression plasmid constructs (pHNd4nB5 and pHNd4nA3) into BL21(DE3)pLysS *E. coli* strain [11] for protein expression, ampicillin–chloramphenicol resistant colonies were selected and cultured in LB media. The culture was induced with 0.4 mM IPTG (isopropylthiogalactoside) when $OD_{600\,nm}$ of the culture reached 0.6–0.8 and was shaken vigorously at 37°C for an additional 3–4 h. The bacterially made nebulin fragments were then prepared from the bacterial cells harvested [12].

2.3. *Immunological verification of the cloned nebulin fragments*

The purified NB5 nebulin fragment which contains 6 of the ~35-residue modules was used to generate specific antibodies in

Balb/c mice. High titer specific anti-NB5 antiserum was obtained after an initial immunization and a single boost. Western blotting using the anti-NB5 antibody and known anti-native nebulin monoclonal antibodies was performed to verify the authenticity of the cloned nebulin fragments expressed in bacteria.

2.4. *Actin binding analysis*

Two types of actin binding assays were performed depending on the solubility behavior of the expressed nebulin fragments in an actin binding buffer (0.1 M KCl, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0). For the more soluble NA3, an F-actin cosedimentation assay [13] was performed. The Coomassie blue-stained gel patterns were digitized and quantitated on a videodensitometer developed at the Clayton Foundation Biochemical Institute (Austin, TX). For the less soluble NB5, a more sensitive antibody-mediated solid-phase actin binding assay was used.

3. RESULTS AND DISCUSSION

3.1. *Expression of human nebulin fragments in* E. coli

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the pelleted bacterial cells containing the expression plasmids (Fig. 1) showed that a very high level of expression (30–32% of the total bacterial protein) of the two cloned human nebulin fragments (NB5 and NA3) was obtained upon IPTG induction. The apparent molecular masses of NB5 (30 kDa) and NA3 (36 kDa) as calculated from gel mobility agreed well with those values derived from predicted amino acid sequences (25936 for NB5 and 31419 for NA3). The expressed nebulin fragments, presented in the form of inclusion bodies, were readily purified in large quantities. Additional characterization, including amino acid analysis and N-terminal peptide sequencing, confirmed that both NB5 and NA3 were coded by the cloned human nebulin cDNA fragments [12].

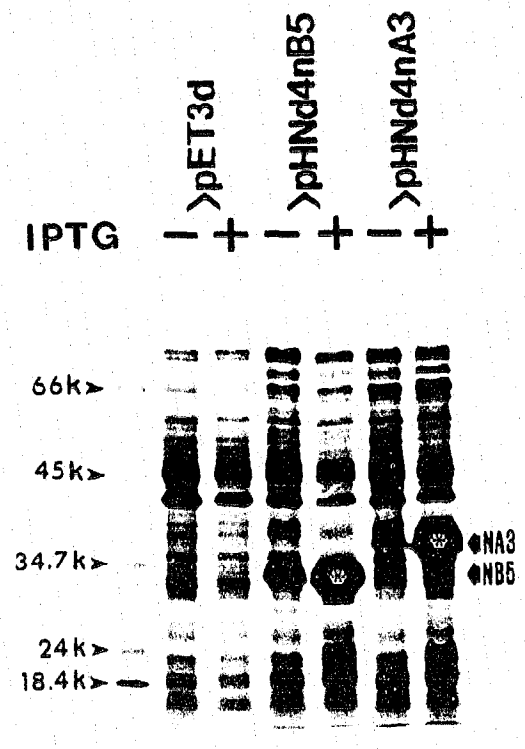


Fig. 1. Expression of human nebulin fragments in *E. coli*. The host bacteria transformed with the vector (pET3d) or the engineered expression plasmids bearing human nebulin cDNA fragments (pHNd4nB5 and pHNd4nA3) were cultured without (lanes marked with −'s) or with IPTG (lanes marked with +'s) induction and the total bacterial proteins were analyzed by 12.5% SDS-PAGE [13]. The results showed a very high-level expression of the two cloned human nebulin fragments NB5 and NA3 (bands labeled with * in the IPTG induced samples).

3.2. *Authenticity of the cloned nebulin fragments*

Western immunoblots on total proteins of human adult and fetal skeletal muscles, as well as several rabbit skeletal muscles (Fig. 2) showed that the anti-NB5 antibody specifically recognized only the nebulin band in all tissues tested. Immunoelectron microscopic localization of NB5 in skeletal muscle [12] revealed that anti-NB5 antibody recognized nebulin in the sarcomere. These results are significant in that they have not only provided a definitive proof that the cloned cDNA with a highly repetitive sequence indeed codes for nebulin but also indicated that the expressed NB5 fragment re-

Fig. 3. Binding of the cloned human nebulin fragments to actin. (A) F-actin co-sedimentation assay. Purified NA3 nebulin fragment (6 μg) and rabbit skeletal muscle F-actin (50 μg or 25 μg) were incubated together or separately in 100 μl of an actin-binding buffer (0.1 M KCl, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0) at room temperature for 1 h. After centrifugation at 120000 × g for 30 min in a Beckman airfuge, the supernatants (lanes S) and pellets (lanes P) were separated and analyzed by 12.5% SDS-PAGE [13]. The results showed that NA3 co-sedimented with F-actin under conditions where NA3 alone was not pelleted. $(A+N)_1$ and $(A+N)_2$ are assays containing actin at 50 μg/100 μl and 25 μg/100 μl, respectively. The gel loading for $(A+N)_1$ was half of that for $(A+N)_2$. (B) Solid-phase actin binding assay. Rabbit skeletal muscle F-actin (100 μg/ml), tropomyosin–troponin complex (Tm·Tn, 100 μg/ml, a kind gift of Dr G.N. Phillips, Rice University), bovine serum albumin (BSA, 10 mg/ml), or skim-milk, each in the actin-binding buffer, was attached to a microtiter plate (Linbro/Titertek, FlowLab., Inc., Mclean, VA) by incubating 100 μl/well at 4°C overnight. The plate was blocked with 0.1% BSA in the same buffer (150 μg/well) at room temperature for 4 h and washed. Purified human nebulin fragment NB5 (100 μl/well, serially diluted from 5×10^{-7} M to 7.8×10^{-9} M in the actin-binding buffer) was added and incubated at room temperature for 2 h followed by two washes. The amount of NB5 bound to the coated actin was determined by a standard ELISA procedure using the mouse anti-NB5 antiserum and peroxidase-conjugated rabbit anti-mouse second antibody (both are diluted in the blocking buffer), and H_2O_2-ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)) substrate [13]. Washings were done with the same actin-binding buffer at room temperature except for the final three washes following second antibody incubation, which was done at 4°C with PBS containing 0.05% Tween-20. The assay was performed in tetrad wells and titration curves were constructed by the average $A_{405\,nm}$ vs NB5 molar concentrations. The result demonstrated a saturable specific binding of NB5 to actin, with no evidence of binding to tropomyosin–troponin complexes, BSA or the milk protein mixture.

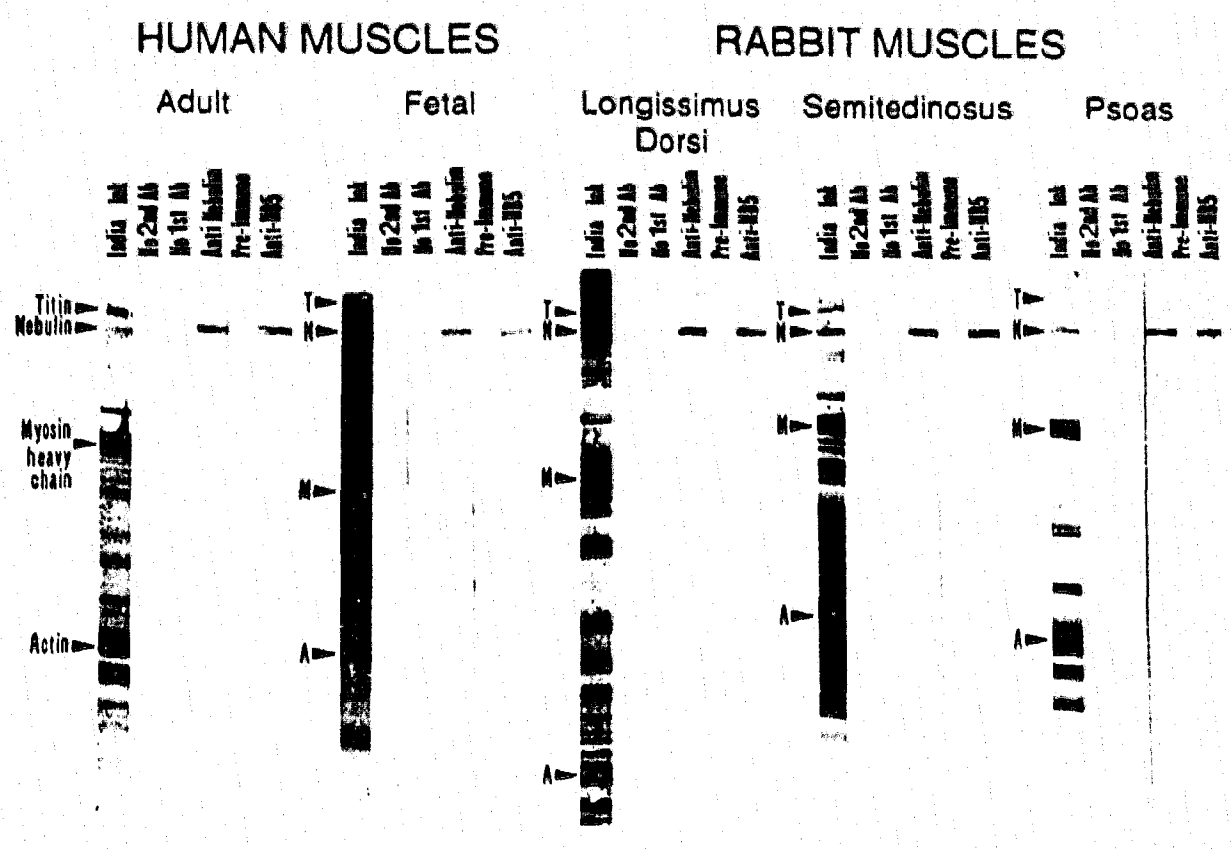


Fig. 2. Immunoblot verification of the authenticity of the cloned human nebulin fragments. Mouse antiserum generated with purified NB5 fragment was used to stain nitrocellulose blots of several human and rabbit skeletal muscles [2]. India ink was used to stain side stripes of each blot to reveal the position of the major sarcomere proteins. In all tissues tested, the anti-NB5 antibody (1 :2000 dilution) specifically recognized the nebulin band which was identified on the same blot by a mixture of anti-nebulin monoclonal antibodies [5]. As shown by the negative controls, no staining was observed when the preimmune serum was tested or either primary or secondary antibody was deleted.

tains the antigenic structures of native nebulin. Furthermore, the presence of this cross-reacting structure in various muscle tissues suggests that NB5 fragment contains common structural elements that are shared among members of this family of giant proteins.

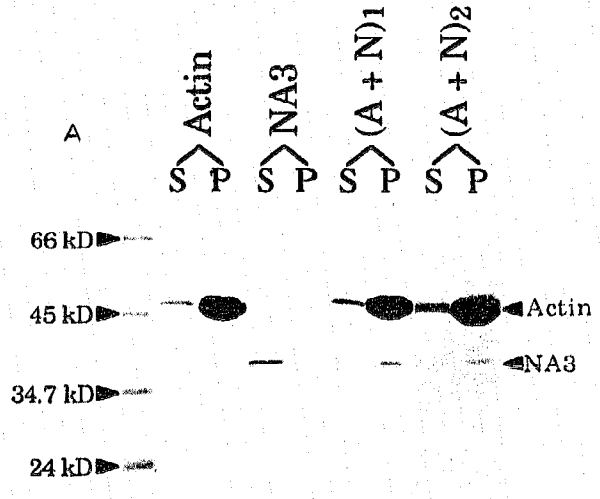
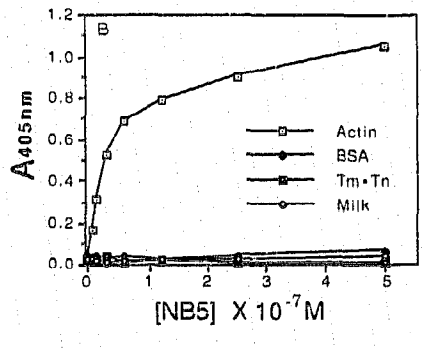


Fig. 3A and B (legend on p. 94).

3.3. *Actin-binding of the cloned nebulin fragments*

Having established that the cloned nebulin fragments are authentic, we examined their actin-binding capacity. Since both nebulin fragments are practically insoluble at neutral pH, a pH 8.0 buffer was used to improve solubility. This actin-binding buffer also contained 10 mM $MgCl_2$ to lower the critical concentration of actin polymerization. For the more soluble fragment NA3, it was feasible to carry out F-actin co-



sedimentation assays. The data in Fig. 3A indicated that NA3 co-sedimented with F-actin under conditions where very little NA3 alone was pelleted. Attempts to determine binding stoichiometry by titration were complicated by the low solubility of NA3 and the need to maintain F-actin above its critical concentration. As estimated by quantitative gel densitometry, the pellets in assays with two different actin concentrations (0.06 mg/ml NA3 mixed with 0.5 mg/ml or 0.25 mg/ml F-actin) contained roughly one NA3 to 17 and 20 actin subunits, respectively. For the less soluble fragment NB5, a more sensitive solid-phase actin binding assay was used instead. The titration curves (Fig. 3B) of NB5 on microtiter plate-coated actin, tropomyosin–troponin (Tm·Tn), bovine serum albumin (BSA), or milk proteins indicated that NB5 bound only to actin in a saturable manner. Although the stoichiometry and binding constant of the nebulin–actin interaction remain to be quantitated, it is clear from the steep titration curve (Fig. 3B) that the binding affinity must be high under this assay condition.

In conclusion, we have demonstrated for the first time that nebulin is an actin-binding protein. The fact that whole nebulin molecule contains the repeating-module sequence of at least 30 times the length of either NA3 or NB5, strongly suggests that nebulin contains a large number of actin binding sites along its length. It is thus conceivable that the skeletal muscle thin filament may require nebulin either as a template for assembly or as a scaffold for stability. Studies of cloned nebulin fragments consisting of defined numbers and types of the ~35 residue modules are in progress to provide further insights into the nature of its binding sites for actin, α-actinin [6], calmodulin [14], as well as the proposed functions of this protein which constitutes a fourth filament of skeletal muscle sarcomere.

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