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Evidence that nebulin is a protein-ruler in muscle thin filaments

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Partial amino acid sequence was obtained from the massive myofibrillar protein nebulin. This consists of repeating motifs of about 35 residues and super-repeats of $7 \times 35 = 245$ residues. The repeat-motifs are likely to be largely α-helical and to interact with both actin and tropomyosin in thin filaments. Nebulin from different species was found to vary in size in proportion to filament length. The data are consistent with the proposal that nebulin acts as a protein-ruler to regulate precise thin filament assembly.

Nebulin; Striated muscle; Thin filament; Protein-ruler

1. INTRODUCTION

Nebulin is a massive protein abundant in vertebrate skeletal muscle (mass $\sim 8 \times 10^5$ [1,2]). Its properties, role and exact location are not known, but antibody labelling suggests an association with thin filaments [3,4] where it has been speculated to act as a 'protein-ruler' controlling filament length [3]. Electron microscopy shows that in many muscles thin filaments have constant length, indicating they are assembled from exact numbers of actin, tropomyosin and troponin subunits. Since the filament is many times longer than these molecules, the observed length precision is difficult to explain without a template or ruler spanning the entire structure. A similar model involving another giant protein, titin (mass $\sim 3 \times 10^6$), has been put forward to account for the precise assembly of myosin into thick filaments [3,5]. Here we describe data supporting the idea that nebulin acts as a thin filament protein-ruler. A partial amino acid sequence indicates an intimate association with thin filaments and nebulin molecules from muscles of different species vary in size in proportion to filament length.

2. MATERIALS AND METHODS

2.1. *cDNA sequencing and PCR*

The cDNA insert of human nebulin clone pHNNS4-3 [6] was subcloned into mp18 [7] and the nucleotide sequence determined by the dideoxy-method [8]. Only one open reading frame was predicted (UWGCG [9] Map program). Rabbit nebulin sequence was obtained by PCR using pairs of 30meric oligonucleotides derived from the human phnns4-3 sequence and rabbit psoas muscle cDNA [10]. Pairs that flanked not more than 7 repeat-motifs were found to amplify efficiently sequences from both species. The sequence from such a fragment comprising 597 bases was obtained. The human and rabbit nebulin nucleotide sequences have been submitted to the EMBL data library under accession numbers X58122 and X58123 respectively.

2.2. *Protein gels and antibody production*

High molecular weight muscle proteins were monitored on 3–10% SDS/polyacrylamide gradient gels [5]. Antibodies were raised to nebulin by immunising a goat with the denatured rabbit protein purified by gel filtration chromatography in SDS [11]. Other methods used are described in [5].

3. RESULTS AND DISCUSSION

Short partial nebulin sequences have been reported, derived from human cDNA clones identified by specific antibodies that label muscle in the thin filament region [1,6]. Further data have now been obtained from one of these clones, pHNNS4-3 [6]. Figure 1 is a graphical self-comparison of 560 residues derived from the enlarged pHNNS4-3 sequence showing it to consist of a repeated motif containing about 35 amino acid residues. The presence of a stronger line every 245 residues indicates that there is also a super-repeat every seven repeat-motifs.

Figure 2 shows alignments of the repeat-motifs and demonstrates that roughly one quarter of the residues they contain are conserved. In the 245 residue super-repeat this figure rises to approximately 70%. The most conserved features of the repeat-motifs are 3 pairs of residues, PD at the beginning of each motif and SD and YK spaced 2 residues apart in the middle. Figure 3 shows comparative data from human and rabbit demonstrating a high degree of conservation between

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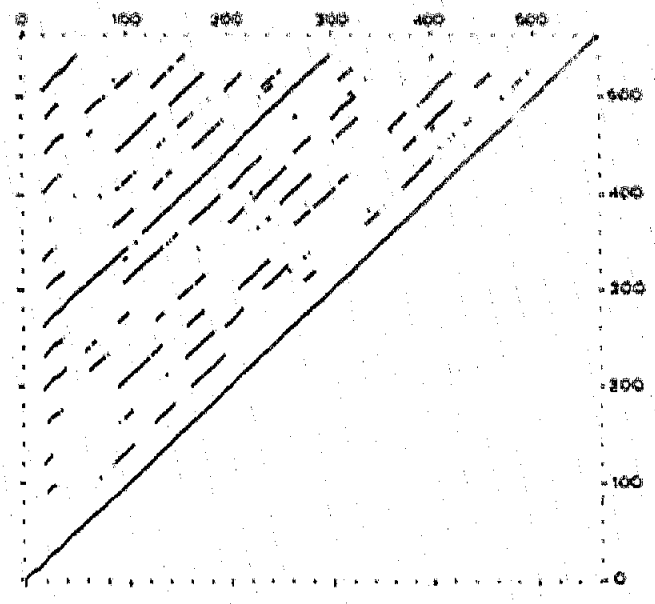

Fig. 1. Repeat and super-repeat structure of nebulin. Two complete super-repeats and the beginning of a third are shown. The sequence derived from the enlarged pHNNS4-3 was compared to itself with the UWGCG [9]. Compare program using a window of 35 residues and a stringency of 15.

species. Between 200 residues in the human and rabbit molecules there are only 9 substitutions and 5 of these are conservative. At the nucleotide level the degree of conservation is slightly less (92% vs 96%, data not shown). Thus almost all the residues in the common sequences have a particular role, and by implication the molecule has an important function. The individual repeat-motifs and their consensus sequences were used to search the NBRF database (Version 26.0) using the UWGCG [8] Wordsearch and Profile programs. No significant matches were found. Analysis of the sequence suggests that the repeat-motifs are largely α-helical, although the N-terminal 5 or 6 residues may have a different conformation. The pattern of conserved residues shows a periodicity indicative of α-helices and the α-helix breakers P and G are rare in the middle of the repeat-motifs. However, there is no heptad repeat of hydrophobic residues [12] showing that the molecule is not dimerised in a coiled-coil.

The 7 motif super-repeat is consistent with the proposal [3,4] that nebulin is associated with thin filaments, since there is one molecule each of tropomyosin and troponin for every 7 actin subunits in the filament. This suggests that the repeat-motifs interact directly with actin subunits. The most likely arrangement is nebulin molecules orientated along the long-pitch helices of the actin, with the 245 residues of the super-repeat spanning 38.5 nm along the filament, similar to tropomyosin [13]. This would produce an axial translation of 0.16 nm per residue, close to the value of 0.15 for a continuous α-helix. This discrepancy is removed if the first few residues in each repeat motif (containing the conserved PD) have a more extended conformation.

Since there is no seven-subunit repeat in F-actin alone, nebulin is also likely to interact with troponin or tropomyosin. A binding site for either would appear as a conserved feature once per super-repeat. One such feature is the sequence KGIGW at the end of motif 1 in each super-repeat (Fig. 2). The generally high degree of conservation between super-repeats also suggests that interactions are made throughout each 38.5 nm interval and the high degree of conservation between species is consistent with this. Since the actin binding sites (possibly SDXXYK) would all be similar and since troponin does not span 38.5 nm, some conserved super-repeat features probably involve interactions with tropomyosin.

Taken together, the sequence data suggest that

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S1.R1   T I D A M S L E H T R N V N Q I Q S D N V Y K D E Y N S F L K G I G W I
S2.R1   - P D A L N V E H S R N A M Q I Q S D N L Y K S D F T N W M K G I G W V
S3.R1   - P D A M S F E L A K N M M Q I Q S D N Q Y K A D Y A D F M K G I G W L
S1.R2   P I G S L E V E K V K K A G D A L N E R K Y R Q H P D T V K F T S V
S2.R2   P I E S L E V E K A K K A G E I L S E K K Y R Q H P E K L K F T Y A
S1.R3   - P D S M G M M L A Q H N T K Q L S D L N Y K V E G E K L K H K Y T I D
S2.R3   - M D T M E Q A L N K S N K L N M D K R L Y T E K W N K D K T T I H V M
S1.R4   - P E L P Q F I Q A K V N A L N M S D A H Y K A D W K K T I R K G Y D L R
S2.R4   - P D T P D I L L S R V N Q I T M S D K L Y K A G W E E E K K K G Y D L R
S1.R5   - P D A I P I V A A K S S R N I A S D C K Y K E A Y E K A K G K Q V G F L S
S2.R5   - P D A I A I K A A R A S R D I A S D Y K Y K K A Y E Q A K G K H I G F R S
S1.R6   L Q D D P K L V H Y M N V A K I Q S D R E Y K K G Y E A S K T K Y H T
S2.R6   L E D D P K L V H F M Q V A K M Q S D R E Y K K G Y E K S K T S F H T
S1.R7   P L D M V S V T A A K K S Q E V A T N A N Y R Q S Y H H Y T L L
S2.R7   P V D M L S V V A A K K S Q E V A T N A N Y R N V I H T Y N M L
Con.    - P D - @ - @ - - - + - - - - @ - S D - - Y + - - @ - - @ + - - - - - -
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Fig. 2. Aligned repeat-motifs of nebulin sequence. Sections of the derived sequence were aligned by eye and no insertions or deletions were found. Residues conserved >50% between repeat-motifs are boxed (Prettyplot, P. Rice, EMBL). S1–3 refer to respective super-repeats and R1–7 the motif numbers within each super-repeat. The characters +, − and @ in the consensus (Con.) line denote charge (positive and negative) and hydrophobicity.

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RAB    1 ANKLNMDKRLYTKKNDKDKTTIHVMPDTPDILLSRVNQITMSDKLYRAGW 50
         .||||||||||||.||:|||||||||||||||||||||||||||||:|||
HUM  341 SNKLNMDKRLYTEKNNKDKTTIHVMPDTPDILLSRVNQITMSDKLYKAGW 390

RAB   51 EEEKKKGYDLRPDAISIKAAKASRDIASDYKYKQAYEQAKGKHIGFRSLE 100
         ||||||||||||||||.||||:||||||||||||||.||||||||||||||
HUM  391 EEEKKKGYDLRPDAIAIKAARASRDIASDYKYKKAYEQAKGKHIGFRSLE 440

RAB  101 DDPKLVHFMQVAKMQSDREYKKAYEKSKTSFHTPVDMLSVVAAKKSQEVA 150
         ||||||||||||||||||||||||:|||||||||||||||||||||||||
HUM  441 DDPKLVHFMQVAKMQSDREYKKGYEKSKTSFHTPVDMLSVVAAKKSQEVA 490

RAB  151 TNANYRNVIHTYNMLPDAMGFELAKNMMQIQSDNQYKADYADFMKGIGW 199
         ||||||||||||||||||||||:|||||||||||||||||||||||||||
HUM  491 TNANYRNVIHTYNMLPDAMSFELAKNMMQIQSDNQYKADYADFMKGIGW 539
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Fig. 3. Comparison of nebulin sequence from rabbit and human. At the nucleotide level the conservation was 92% (UWGCG) [9] Bestfit program). Most differences were in the third codon position. The similarity at the peptide level was therefore greater (96% matching exactly, 98% scoring conservative substitutions as matches).

nebulin consists of a series of discrete α-helical domains. These may bind to successive actin subunits along the long-pitch helices of the thin filament and also interact with tropomyosin. The screw symmetry of the filament necessitates 2 or a multiple of 2 nebulin molecules per filament. The amount of nebulin present in muscle (-4% of myofibrillar protein) is consistent with up to 4 molecules per filament [3], but 2 seems most likely. It is not surprising that nebulin has not been seen directly in 3D reconstructions of native thin filaments from electron micrographs [14,15], since single α-helical strands have a width ~ 1 nm, which is below the resolution of the reconstructions.

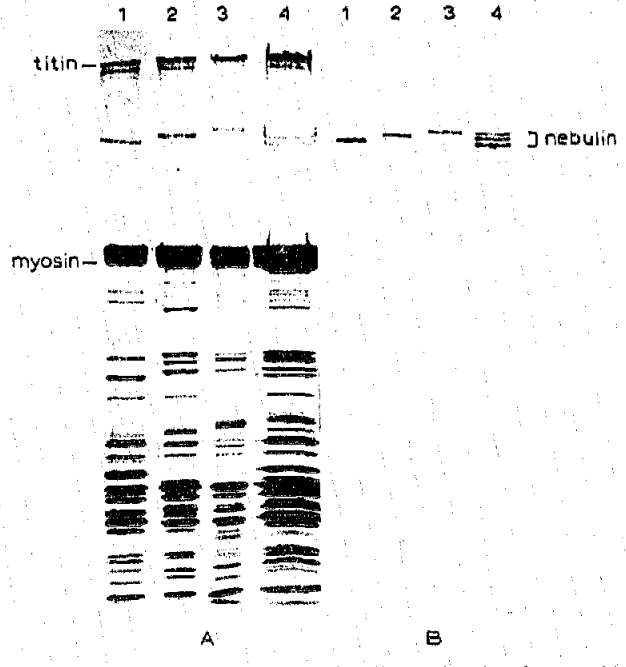

Fig. 4. Size differences between nebulin molecules from rabbit, chicken and beef muscle. (A) Coomassie blue-stained gradient gel. Lanes 1, 2 and 3 show whole fresh muscle from beef (psoas), chicken (pectoralis major) and rabbit (psoas), respectively. Lane 4 is a combination of the 3. (B) Western blot of same gel probed with nebulin antiserum. See also [22] and [23] for indications of nebulin size variation in other muscles and species.

The close association with thin filaments implied by the sequence data is consistent with the proposal that nebulin acts as a protein-ruler to regulate assembly in filaments containing exact numbers of subunits. The simplest model is a pair of parallel nebulin molecules independently spanning the length of each filament. Nebulin would thus assist actin polymerisation and assembly would stop when the end of the nebulin molecule was reached. The molecular weight of nebulin is not accurately known, but the estimated value of 700–800 kDa [1,2] would result in a molecular length of roughly 1 μm if entirely α-helical, which is comparable to the length of the filament. Wang and co-workers have recently made a preliminary report that approximately 80% of the sequence of nebulin consists of 35 and 240 residue repeats and super-repeats [16]. Antibody labelling data suggest single nebulin molecules span most if not all of the filament [17] and there is evidence they extend into the Z-line to interact with α-actinin [18].

One prediction of the protein-ruler hypothesis is that in exactly specified filaments having different lengths, the size of nebulin molecules should vary proportionately. Consistent with this, Fig. 4 shows SDS/polyacrylamide gels of skeletal muscles from rabbit, chicken and beef muscle, where filament length is 1.05 μm [19], 1.1 μm [20] and 1.3 μm [19] respectively. Titin and myosin from these muscles have constant size, but the nebulin bands have mobilities which vary roughly in proportion to filament length. Also compatible with the protein-ruler hypothesis is the fact that in muscles where thin filament length is not precisely specified, such as cardiac, nebulin is absent [3,21].

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