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Characterization of amphioxus nebulin and its similarity to human nebulin

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SUMMARY

Identification of a large molecule in muscle is important but difficult to approach by protein chemistry. In this study we isolated nebulin cDNA from the striated muscle of amphioxus, and characterized the C-terminal regions of nebulins from other chordates. Although the sequence homology with that of human is only 26%, the C-terminal region of amphioxus nebulin has similar structural motifs of 35 amino acid nebulin repeats and an SH3 domain. Using *in situ* indirect immunofluorescence analysis with a specific antibody raised to the bacterially produced recombinant peptide, we identified that this nebulin fragment is located in the Z-line of the sarcomere, similar to human nebulin. Pull-down and co-sedimentation assays *in vitro* showed that the C-terminal region binds to actin, α-actinin and connectin (titin). These results suggest that the C-terminal region of amphioxus nebulin plays a similar role in maintaining striated muscle structure to that of human nebulin. This is the first report of the exact location of nebulin in amphioxus muscle.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/5/668/DC1

Key words: α-actinin, actin, chordate, connectin, muscle.

INTRODUCTION

Nebulin is a large, 773 kDa protein found in vertebrate skeletal muscles (Wang, 1982; McElhinny et al., 2003). The primary structure of human nebulin consists of a N-terminal glutamic-acid-rich domain, followed by 185 (M1–185) contiguous nebulin repeats of approximately 35 amino acids with a central SDXXYK consensus sequence, a serine-rich region and a C-terminal Src homology 3 (SH3) domain (Labeit and Kolmerer, 1995). Nebulin repeats (M9–162) in the I-band region of the sarcomere consist of sets of seven-repeats. These seven-repeats comprise approximately 245 amino acid residues (i.e. 35 amino acid residues per repeat × seven repeats) to form a super-repeat; there are 22 super-repeats (SR1–22).

Within the sarcomere, the N-terminal region is located in the pointed end of the thin filaments, the central region along the thin filaments and the C-terminal region in the Z-line (Wang and Wright, 1988). Binding assays have revealed that each nebulin repeat (SDXXYK) binds to actin (Chen et al., 1993; Lukoyanova et al., 2002). Furthermore, nebulin repeats 1–3, repeat 163–170, repeat 185–SH3 domain and the SH3 domain bind to tropomodulin, desmin, connectin (also called titin), and myopalladin and β-actinin (also called CapZ), respectively (Bang et al., 2001; Bang et al., 2002; Jin and Wang, 1991; McElhinny et al., 2001; Witt et al., 2006).

Existence of nebulin in invertebrates has not been reported; however, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of extracts of amphioxus striated muscle revealed bands corresponding to the size of nebulin (Hu et al., 1986; Locker and Wild, 1986). In 1997, Kimura et al. (Kimura et al., 1997) reported that one of several nebulin antibodies reacted with a 750 kDa protein found in amphioxus striated muscle, and in 1999, Fock and Hinssen (Fock and Hinssen, 1999) prepared an antibody against this protein and showed that it was located in the I-Z-I region of the sarcomere. However, because these findings could have resulted from a nebulin-mimicking epitope or simply from cross-immunoreactivity with an SH3 domain protein, this 750 kDa protein was not proved to be nebulin.

In this study, for the first time, we cloned the C-terminal region of the 750 kDa protein identified in amphioxus striated muscle, examined its functional properties and compared it to vertebrate nebulin.

MATERIALS AND METHODS

cDNA cloning, sequencing and analysis

Approximately 500 specimens of adult amphioxus (*Branchiostoma belcheri* Gray) were collected in the Enshu-Nada Sea, Japan (Kubokawa et al., 1998). Nerve cord and muscle tissues were used to produce a cDNA library that was constructed in lambda ZAP II (Stratagene, La Jolla, CA, USA) using oligo(dT) primers. The fragments encoding the homologue of nebulin were obtained from the cDNA library using an EST analysis system established by the sequencing laboratory in RIKEN, Kobe, Japan (Mineta et al., 2003). Three clones containing the nebulin homologues were isolated from approximately 7000 clones of the cDNA library. The domains were aligned with reference to Labeit and Kolmerer (Labeit and Kolmerer, 1995). The sequence analysis and homology search were performed using GENETYX-WIN Ver. 4.0.4 (Genetyx, Tokyo, Japan).

SDS-PAGE and immunoblotting

A piece of freshly excised amphioxus striated muscle was dissolved in five volumes of a solution containing 10% SDS, 40 mmol l^{-1} dithiothreitol, 10 mmol l^{-1} EDTA and 100 mmol l^{-1} Tris–HCl (pH 8.0). The solution with the dissolved muscle was boiled for 3 min and clarified by centrifugation at 15,000 ***g*** for 10 min. The supernatant proteins were separated by SDS-PAGE using 2–6% gradient polyacrylamide gels (acrylamide:methylenebisacrylamide, 30:1.5; w/w) or 2–15% gradient polyacrylamide gels (acrylamide: methylenebisacrylamide, 30:0.8; w/w) (Laemmli, 1970).

The protein bands were electrically transferred onto a nitrocellulose membrane (Towbin et al., 1979) and treated with primary antibody and horseradish-peroxidase-conjugated anti-rabbit IgG secondary antibody (Code number: P-0399, Dako, Carpinteria, CA, USA).

Antibody production

The cDNA fragment of amphioxus nebulin (1007–1848 bp of clone 5361) was generated by restriction enzyme digestion (*Sma*I–*Sal*I) and inserted into the *Pvu*II–*Sal*I site of the pGEX4T-3 vector (GE Healthcare, Piscataway, NJ, USA). Recombinant GST-tagged protein was expressed in *Escherichia coli* (*E. coli*) XL1 Blue-MRF' with 3 l of LB + ampicillin culture medium under the conditions of OD_{600}=0.5, IPTG 0.1 mmol l^{-1}, 37°C and 3 h. The bacteria were harvested by centrifugation (1500 ***g***, 4°C, 10 min), dialyzed with 120 ml PBS, treated with 80 ml sample buffer, boiled for 10 min and subjected to 227 gels of 10% SDS-PAGE. The bands of recombinant protein were excised and the recombinant protein was electrically extracted from the gels into a running buffer solution (0.1% SDS, 25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine) at 400 mA for 16 h. The protein was dialyzed with phosphate-buffered saline (PBS), and GST was deleted using thrombin (Sigma-Aldrich Japan, Tokyo, Japan; 1/1000 volume of recombinant protein solution) at 22°C for 16 h. The solution was added to the sample buffer and subjected to 88 gels of 12.5% SDS-PAGE. The nebulin fragment without GST was electrically extracted from the gel, dialyzed with PBS, conjugated with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI, USA) and injected three times (0.25, 0.9 and 0.1 mg protein, respectively) into a rabbit. The antiserum was separated from the blood by centrifugation.

Immunofluorescence microscopy

After skinning the amphioxus specimens and collecting the muscle tissue under a stereoscopic microscope, the myofibers were stretched with relaxing buffer (50 mmol l^{-1} KCl, 10 mmol l^{-1} EGTA, 10 mmol l^{-1} $NaPO_4$, 3 mmol l^{-1} ATP and 0.5% Triton X-100; pH 7.5) and fixed with buffer (3.5% formaldehyde, 45 mmol l^{-1} KCl, 9 mmol l^{-1} EGTA, 9 mmol l^{-1} $NaPO_4$, 2.7 mmol l^{-1} ATP and 0.45% Triton X-100; pH 7.5) for 1 min. The tissue was homogenized five times for 2 s each in PBS containing 0.5 mmol l^{-1} leupeptin using Ultra-turrax T-25 (IKA-Labortechnik, Staufen, Germany) and then fixed on a glass slide. The samples were fixed with 3.7% formaldehyde in PBS for 15 min, washed twice with PBS for 5 min each and blocked with 1% bovine serum albumin (BSA) in PBS for 15 min. The fixed samples were stained with amphioxus nebulin (1:50) and α-actinin A7811 (1:800; Sigma-Aldrich Japan, Tokyo, Japan) antibodies for 12 h, washed three times with PBS for 10 min and reacted with Alexa Fluor 488 (1:2500)- and Alexa Fluor 546 (1:4000)-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). The samples were then washed three times with PBS for 15 min, fixed with 3.7% formaldehyde in PBS for 15 min and washed with PBS for 15 min. Anti-fador was added to the samples and they were covered with a cover glass. Fluorescence was observed with fluorescence microscope (Zeiss Axioskop 2 plus; Carl Zeiss, Oberkochen, Germany).

Protein preparation

The cDNA fragments of amphioxus nebulin repeats 3–9 (AN3–9) and the unique region of the SH3 domain (U–SH3) generated by restriction enzyme digestion were cloned into the pGEX6P series (GE Healthcare, Piscataway, NJ, USA). The recombinant proteins tagged with GST were expressed in *E. coli* BL21 (DE3) pLysS in 250 ml LB containing ampicillin. The conditions were OD_{600}=0.5, IPTG 0.5 mmol l^{-1}, 37°C and 3 h. The bacteria were harvested by centrifugation (1000 ***g***, 4°C, 10 min). The bacteria with the GST-fusion proteins were dissolved in PBS (140 mmol l^{-1} NaCl, 10 mmol l^{-1} Na_2HPO_4, 2.7 mmol l^{-1} KCl and 1.8 mmol l^{-1} KH_2PO_4; pH 7.3), frozen at –80°C for 20 min, sonicated at output 2 for 1 min (TOMY UD-200; Tomy Seiko, Tokyo, Japan) and centrifuged at 14,000 ***g*** for 30 min at 4°C. The supernatant was loaded onto a 2 ml glutathione Sepharose 4B column (GE Healthcare, Piscataway, NJ, USA), washed with PBS and eluted with 10 mmol l^{-1} glutathione (reduced) in 50 mmol l^{-1} Tris–HCl (pH 8.0). For the co-sedimentation assay, GST was deleted from GST–AN3–9 on a column using PreScission Protease (GE Healthcare, Piscataway, NJ, USA) with buffer (50 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA and 1 mmol l^{-1} DTT; pH 7.0) at 4°C for 12 h.

Actin was prepared from acetone powder of rabbit skeletal muscle using the protocol described by Spudich and Watt (Spudich and Watt, 1971). G-actin was polymerized in 0.1 mol l^{-1} KCl. α-Actinin was prepared from rabbit skeletal muscle by the method of Goll et al. (Goll et al., 1972). Connectin was prepared according to Kimura et al. (Kimura et al., 1992).

Co-sedimentation assay

F-actin (2.5 μmol l^{-1}) or BSA (5 μmol l^{-1}) was mixed with AN3–9 (12.5 μmol l^{-1}) in 500 μl buffer (2 mmol l^{-1} Tris, 0.1 mmol l^{-1} $CaCl_2$, 0.1 mol l^{-1} KCl and 0.01% NaN_3; pH 8.0) at 25°C for 1 h. The mixture was centrifuged (15,000 ***g***, 4°C, 30 min), and the supernatant and pellet were diluted with SDS sample buffer and subjected to SDS-PAGE.

GST pull-down assay

GST–AN3–9 (2 μg for α-actinin pull down) or GST–U–SH3 (2 μg for connectin pull-down) were bound to 20 μl glutathione Sepharose 4B beads according to the manufacture's protocol. The beads were washed twice with 150 μl buffer (80 mmol l^{-1} KCl, 2 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Hepes, 1 mmol l^{-1} DTT and 2% Triton X-100; pH 7.3), and bound with α-actinin (4 μg, 20 μl) or connectin (5 μg, 150 μl) in buffer at 4°C for 1 h. They were washed four times with 150 μl buffer, dissolved in 20 μl SDS sample buffer, and subjected to SDS-PAGE. The gels were stained with Coomassie Brilliant Blue.

RESULTS

Primary structure of amphioxus nebulin C terminus

Three clones were obtained from a mixed cDNA library of amphioxus muscle tissues and nerve cord. Sequencing revealed a 2527 bp sequence containing a poly(A)$^+$ tail and a stop codon, suggesting that it contains the C-terminal region of the protein (DDBJ accession no. AB244086). The amphioxus sequence contains 13 nebulin repeats and an SH3 domain with unique regions between these modules (Fig. 1A).

Amphioxus nebulin repeats were assigned the numbers Neb-1–13 for descriptive purposes. The consensus sequence observed in each nebulin repeat was a PEXXRXKXVXKIQ motif in the N-terminal region and a GKXYTXVXDT/D motif in the C-terminal region, as well as a SE and a Y in the SEXXYX motif (Fig. 1B,C). P1, E2, R5 and K7 in the N-terminal and S14 and Y18 in the SEXXYX motif of the amphioxus nebulin repeats correspond to the human C-terminal nebulin repeats (M172–185; Fig. 1C). The SSVLYKEN box, which is present in human M174–181, was not found in the amphioxus nebulin repeats.

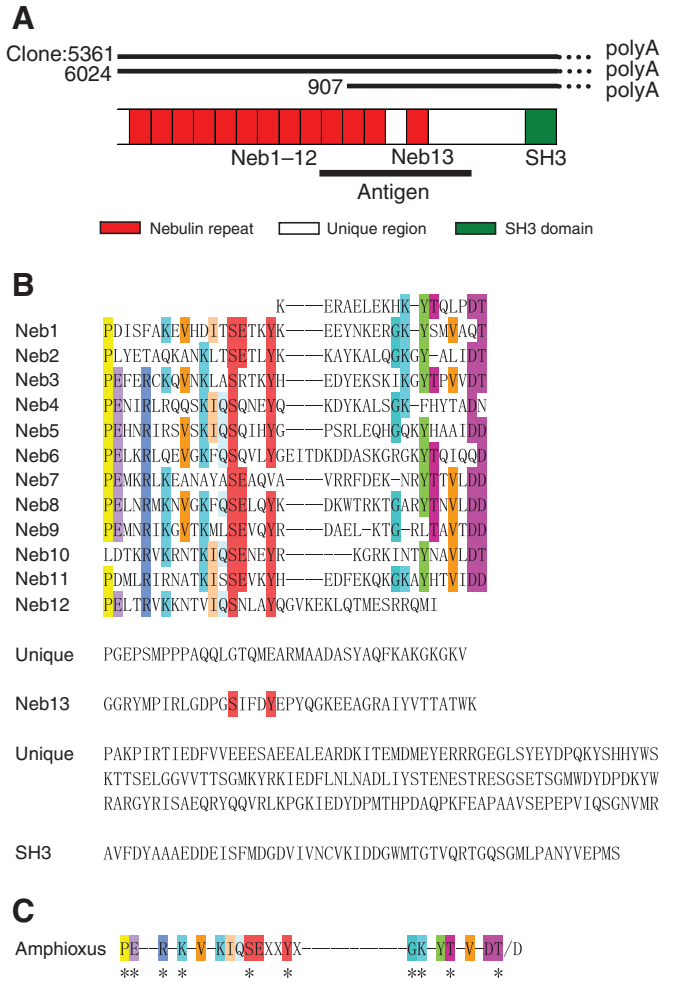

Fig. 1. Domain structures and primary structure of the nebulin cDNA C-terminal region in amphioxus striated muscle. (A) Domain structures. Thin black bars at the top show the size of each clone and the thicker bar below indicates the position of the Pc AmpN antigen. (B) Amino acid sequence of amphioxus nebulin. (C) Consensus sequences of amphioxus and human nebulin repeats.

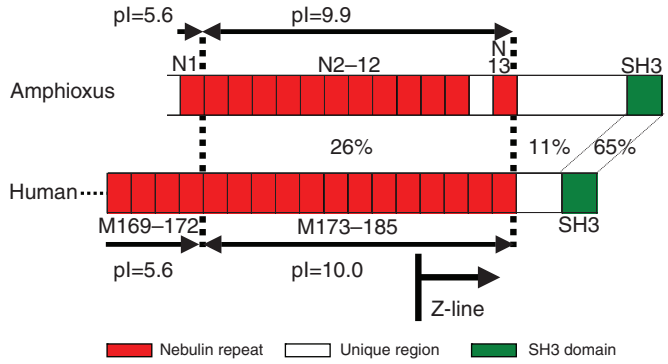

Fig. 2. Comparison of the C-terminal region of amphioxus and human nebulins. The domain structure and isoelectric point (pI) are presented according to Labeit and Kolmerer (Labeit and Kolmerer, 1995), and the I-Z border is presented according to Millevoi et al. (Millevoi et al., 1998). Amphioxus, amphioxus nebulin; Human, human nebulin.

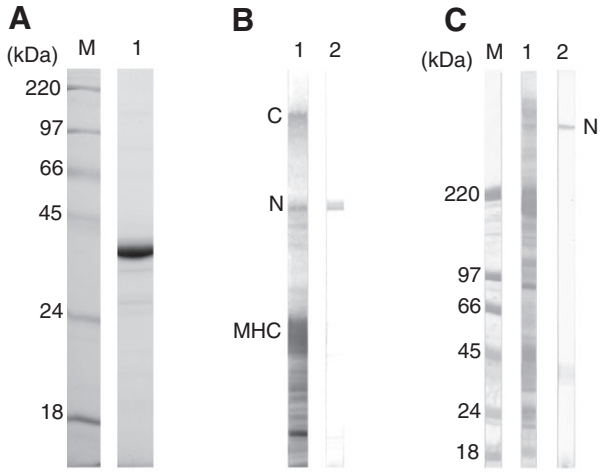

Fig. 3. SDS-PAGE and immunoblot detection of amphioxus striated muscle. (A) Antigen for amphioxus nebulin. M, molecular size markers; lane 1, Pc AmpN antigen. (B,C) Immunoblot detection of amphioxus striated muscle using Pc AmpN (thick bar in Fig. 1A). Electrophoresis was performed on 2–6% (B) or 2–15% (C) polyacrylamide gradient gels. M, molecular size marker; lane 1, amido black stains; lane 2, immunoblot detection by Pc AmpN. C, connectin; N, nebulin; MHC, myosin heavy chain.

Comparison between amphioxus and vertebrate nebulins

The amphioxus nebulin repeats shared an approximately 26% homology with human C-terminal nebulin repeats. We predicted the secondary structure of the amphioxus nebulin repeat region using the Multivariate Linear Regression Combiner secondary structure prediction program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_mlrc.html). The secondary structure consisted of 48% α-helix, 0% β-structure and 47% random coil. The composition was similar to that of the secondary structure of the C-terminal region of the human nebulin repeat (41% α-helix, 0% β-structure and 52% random coil) predicted using the same program. Calculation of the isoelectric points (pIs) based on the amphioxus C-terminal amino acid sequence revealed pIs of 5.6 and 7.1–10.8 (total pI=9.9) for Neb-1 and Neb-2–13, respectively, indicating that the pIs of the amphioxus nebulin repeats are in accordance with those of the human nebulin repeats (pI=5.6 for M172 and pI=10.0 for M173–185) (Labeit and Kolmerer, 1995) (Fig. 2).

The PGSIFDYEP of the last nebulin repeat (Neb-13) in amphioxus (Fig. 1B) is conserved across species, and corresponds to the last human nebulin repeat M185 (X83957) (Labeit and Kolmerer, 1995), chicken nebulin repeat M65 (AB024330) (Suzuki et al., 2000), the zebrafish nebulin-like protein (AL974314) and the nebulin-like protein (nebulette) of vertebrate cardiac muscle (Y16350) (Millevoi et al., 1998; Moncman and Wang, 1995). Furthermore, when we compared the C-terminal SH3 domain of amphioxus nebulin to human (X83957), chicken (AB024330) and zebrafish (AL974314), we observed a high homology (approximately 65%) with human nebulin (Fig. 2), revealing that the amino acid sequence of the SH3 domain was well conserved across species (Fig. 2). However, two unique regions, which are between Neb-12 and Neb-13 and between Neb-13 and the SH3 domain, showed no homology with any region of the human nebulin (Fig. 2).

The domain structure, secondary structure and the pIs of the C-terminal region of amphioxus nebulin are similar to those of vertebrate nebulin.

Localization of the amphioxus nebulin C-terminal region in the sarcomere

To confirm that the cDNA was from amphioxus nebulin and to determine the position of the C-terminal region within the sarcomere, we prepared an antibody (PcAmpN) using the recombinant protein from Neb 10 to the former half of the unique region as an antigen (Fig. 1A, Fig. 3A). Immunoblot analysis showed that the antibody reacted only with the 750 kDa band and not with other proteins such as nebulette (107 kDa; Fig. 3B,C). This confirmed the sequence to be that of amphioxus nebulin cDNA.

Double immunostaining of amphioxus myofibrils with PcAmpN and an α-actinin monoclonal antibody revealed that the C-terminal region of amphioxus nebulin is localized to the Z-line of the sarcomere and co-localizes with α-actinin (Fig. 4).

Binding of amphioxus nebulin to F-actin, α-actinin and connectin

It is known that vertebrate nebulin is localized along thin filaments and the nebulin repeats bind to actin. To clarify whether amphioxus nebulin repeats bind to actin, we examined the binding of nebulin repeats 3–9 to F-actin by far-western blot analysis and a co-sedimentation assay. The far-western blot analysis showed that F-actin binds to the amphioxus nebulin repeats, similar to human nebulin repeats (supplementary material Fig. S1B). Furthermore, the results of the co-sedimentation assay showed that F-actin alone did not precipitate after centrifugation at 15,000 ***g*** for 30 min, but it co-precipitated with the nebulin repeats, indicating that amphioxus nebulin repeats bind to F-actin (Fig. 5).

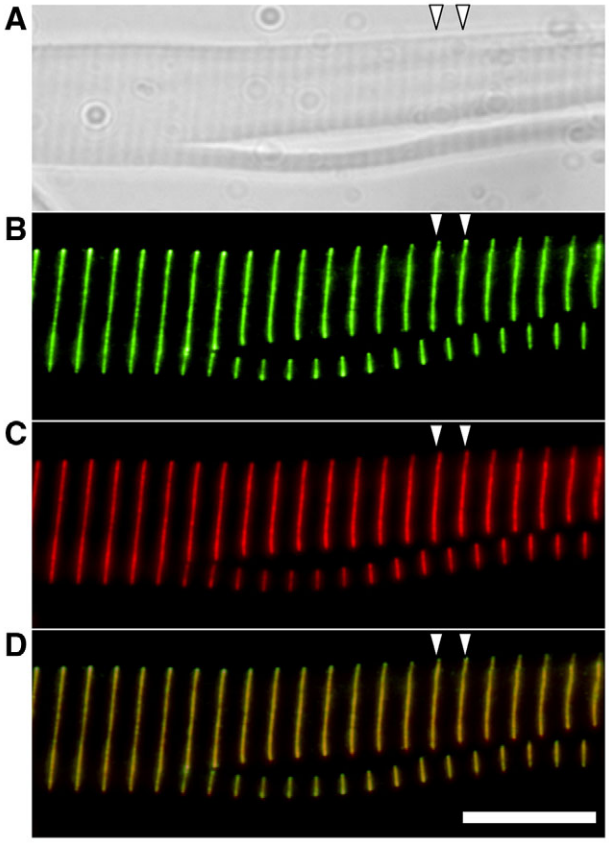

Fig. 4. Immunofluorescence microscopic observations of amphioxus striated muscle sarcomeres. (A) Phase-contrast image. (B,C) Immunostaining with Pc AmpN (B) and α-actinin (C) monoclonal antibodies. (D) Merged image of B and C. Arrowheads, Z-line. Scale bar, 10 μm.

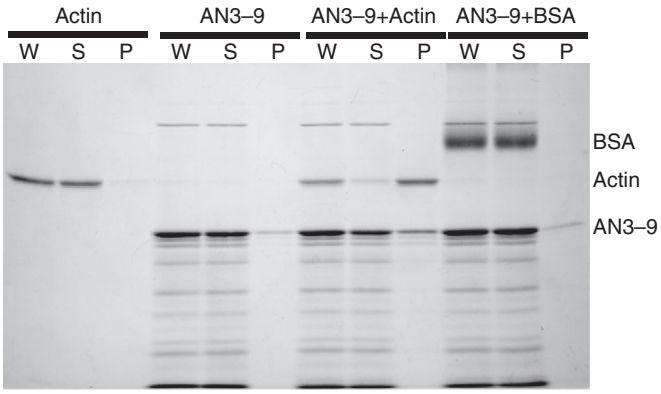

Fig. 5. Binding of amphioxus nebulin and F-actin examined by co-sedimentation. Actin, actin only; AN3–9, amphioxus nebulin repeats 3–9 dissolved in GST; AN3–9+Actin, amphioxus nebulin repeats 3–9 +actin; AN3–9+BSA, amphioxus nebulin repeats 3–9 +BSA. W, before centrifugation; S, supernatant after centrifugation; P, pellet after centrifugation. Electrophoresis was performed on a 12.5% polyacrylamide gel.

Next, we examined binding of the amphioxus nebulin repeats with purified α-actinin by far-western blot analysis and a GST pull-down assay. The results of the far-western blot analysis showed that α-actinin binds to nebulin repeats 3–9 (supplementary material Fig. S1C). Furthermore, the results of the GST pull-down assay showed that α-actinin precipitated when added to the GST-fusion protein with nebulin repeats 3–9 (Fig. 6, lane 5), but not when it was added to GST alone (Fig. 6, lane 6). The results of a control experiment showed that amphioxus nebulin repeats 3–9 do not bind to BSA (supplementary material Fig. S2).

We examined binding of the SH3 domain from the unique region (U–SH3) in amphioxus nebulin with purified rabbit connectin by far-western blot analysis and a pull-down assay. The results of the far-western blot analysis showed that connectin binds to the U–SH3 domain (supplementary material Fig. S1D). Furthermore, the result of the GST pull-down assay revealed that connectin binds to the U–SH3 domain of amphioxus nebulin, but not to GST alone (Fig. 7).

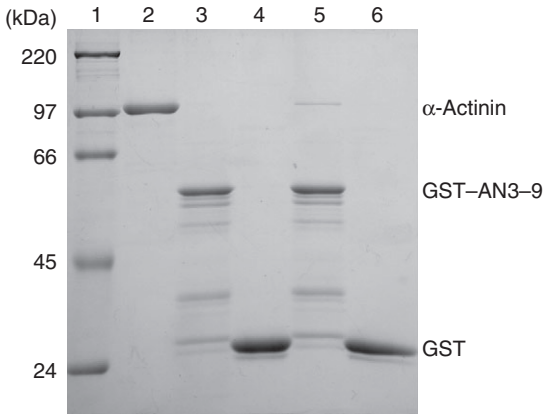

Fig. 6. Binding of amphioxus nebulin and α-actinin, examined by a GST pull-down assay. Lane 1, molecular size marker; lane 2, α-actinin used for the pull-down assay; lanes 3–6, after the pull-down assay: lane 3, GST fusion protein for amphioxus nebulin repeats 3–9 –α-actinin; lane 4, GST only –α-actinin; lane 5, GST fusion protein for amphioxus nebulin repeats 3–9 +α-actinin; lane 6, GST only +α-actinin. GST–AN3–9GST, fusion protein for amphioxus nebulin repeats 3–9. Electrophoresis was performed on a 10% polyacrylamide gel.

DISCUSSION

The present study elucidated the primary structure, localization and binding proteins of the C-terminal region of amphioxus nebulin. These results confirm earlier findings that nebulin is expressed in amphioxus and suggest that it plays a role similar to that of vertebrate nebulin.

In human nebulin, repeats M167–172 are negatively charged and repeats M173–185 are positively charged under physiological conditions. Similarly, in amphioxus nebulin, Neb-1 is negatively charged and Neb-2–13 are positively charged. Thus, the charge transition is conserved between human and amphioxus nebulins. Although the function of the charge transition region is unknown, its localization within or around the Z-line suggests that it is involved in the formation and structural maintenance of the Z-line.

α-Actinin is the main component of the Z-line of the sarcomere, and is also the location of the N terminus of connectin (titin) (Tskhovrebova and Trinick, 2003). We showed that amphioxus nebulin bind to α-actinin and connectin (titin) and that the C-terminal region of amphioxus nebulin is localized in the Z-line of the sarcomere (Fig. 4). These results suggest that amphioxus nebulin maintains the structure of the Z line by binding to α-actinin and connectin, similar to the C-terminal region of human nebulin.

We predicted that the secondary structure of the amphioxus nebulin repeat region was 48% α-helix and 0% β-structure. The secondary structure was similar to that of the C-terminal region of the human nebulin repeat. This is because both amphioxus and human nebulins have the consensus sequences PEXXRXK at the N terminus, SXXXYX in the middle and GKXXTXXXXT at the C terminus of each repeat. As a result, amphioxus nebulin functions in a similar manner to human nebulin, even though there is only a 26% homology at the sequence level. This is because the helical part of the protein can present the conserved surface and interact to other proteins (McLachlan and Karn, 1982).

The thin filaments in the striated muscle of arthropods and mollusks do not have a uniform length of 1 μm as in vertebrate skeletal muscle, whereas the thin filaments of amphioxus striated muscle have a uniform length of 1 μm (Hagiwara et al., 1971). Although amphioxus is not a vertebrate and is the phylogenetically lowest chordate, its nebulin functions similarly to vertebrate nebulin, which might explain the uniform 1 μm size of the thin filaments in amphioxus striated muscle. Taken together with our result of the interaction of amphioxus nebulin with actin, α-actinin and connectin, the thin filaments in amphioxus striated muscle might be maintained in a manner identical or similar to that of the thin filaments of vertebrate skeletal muscle.

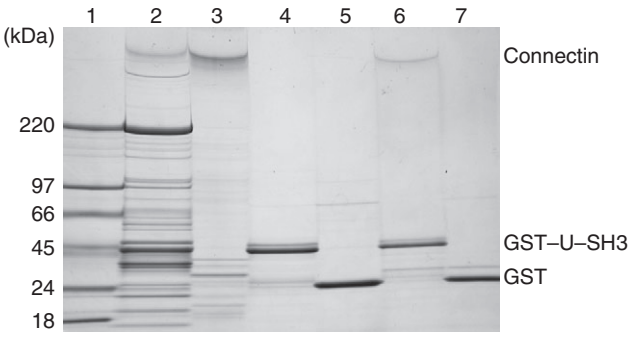

Fig. 7. Binding of amphioxus nebulin and connectin examined by a GST pull-down assay. Lane 1, molecular size marker; lane 2, rabbit skeletal muscle; lane 3, connectin used for the pull-down assay; lanes 4–7, after the pull-down assay: lane 4, GST–U–SH3 –connectin; lane 5, GST only –connectin; lane 6, GST–U–SH3 +connectin; lane 7, GST only +connectin. GST-U–SH3, GST fusion protein for amphioxus nebulin U–SH3 region. Electrophoresis was performed on a 2–15% polyacrylamide gel.

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