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Isolation of a cDNA Encoding the Motor Domain of Nonmuscle Myosin Which Is Specifically Expressed in the Mantle Pallial Cell Layer of Scallop (*Patinopecten yessoensis*)

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It has been reported that catch and striated muscle myosin heavy chains of scallop are generated through alternative splicing from a single gene [Nyitray *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91, 12686–12690]. They suggested that the catch muscle type myosin was expressed in various tissues of scallop, including the gonad, heart, foot, and mantle. However, there have been no reports of the primary structure of myosin from tissues other than the adductor muscles. In this study, we isolated a cDNA encoding the motor domain of myosin from the mantle tissue of scallop (*Patinopecten yessoensis*), and determined its nucleotide sequence. Sequence analysis revealed that mantle myosin exhibited 65% identity with *Drosophila* non muscle myosin, 60% with chicken gizzard smooth muscle myosin, and 44% with scallop striated muscle myosin. The mantle myosin has inserted sequences in the 27 kDa domain of the head region, and has a longer loop 1 structure than those of scallop striated and catch muscle myosins. Phylogenetic analysis suggested that the mantle myosin is classified as a smooth/nonmuscle type myosin. Western blot analysis with antibodies produced against the N-terminal region of the mantle myosin revealed that this myosin was specifically expressed in the mantle pallial cell layer consisting of nonmuscle cells. Our results show that mantle myosin is classified as a nonmuscle type myosin in scallop.

Key words: cloning, mantle, nonmuscle myosin, pallial cell layer, scallop.

Myosin of scallop adductor muscle has two kinds of light chains, SH-light chains and regulatory light chains (RLC). EDTA treatment of scallop muscle myosin causes reversible removal of RLC with accompanying loss of Ca-sensitivity, which is characteristic of scallop muscle myosin (*1*). Unlike skeletal muscle myosin, smooth muscle, and scallop muscle myosins are regulated molecules in muscle contraction. Scallop muscle contraction is regulated through direct binding of calcium to myosin RLC (*2*), and smooth muscle contraction is triggered by phosphorylation of myosin RLC.

Scallop adductors contain two different types of muscles, striated and catch muscles. Catch muscle is unique in that it is capable of maintaining contractive tension for a long time with a very low metabolic turnover (catch contraction). Although the mechanism underlying catch contraction is not clear, the contribution of paramyosin (*3*, *4*), phosphorylation of myosin heavy chains (*5*), and RLC (*6*) has been suggested. These unique properties of scallop have led to extensive studies on scallop striated and catch muscle myosins.

The scallop striated and catch muscle myosins arise through alternative RNA splicing of a single myosin heavy chain gene (*7*). A RT-PCR study involving alternative exon-specific primers showed that catch muscle myosin is expressed in the mantle, heart, and other tissues, but not in striated muscle (*7*). However, there have been no reports of the sequence of myosin from tissues other than muscle. The mantle tissue of bivalve molluscan shells consists of a muscle portion and nonmuscle portions such as mucous cells and epithelial cells. The epithelial cells are known to play roles in such as secretion of the shell organic matrix proteins (*8*, *9*), and migration and proliferation during the wound healing process (*10*). It is expected that various types of myosins may play roles during motile events of the epithelial cells. Therefore, we tried to isolate a cDNA encoding myosin from the mantle tissue including epithelial cells.

In the present study, we isolated a cDNA encoding the head domain of myosin from the mantle tissue including the pallial cell layer of scallop.

MATERIALS AND METHODS

RT-PCR—Total RNA was extracted from the mantle tissue including the pallial cell layer with guanidinium isothiocyanate and then purified. Aliquots (1 μg) of total RNA were reverse-transcribed with oligo(dT) primers, and PCR was conducted with a set of degenerate ATP binding site primers having the following sequences described by Bemet *et al.* (*11*): 5′-GGIGARWSIGGIGCIGGIAARAC-3′ as a sense primer and 5′-GTYTTIGCRTTICCRAAIGCYTC-3′ as an antisense primer. After amplification, the products were separated on a 3% low melting agarose gel (Nusieve GTG

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS-PAGE, PAGE in the presence of SDS; GST, glutathione-S-transferase; RLC, regulatory light chain; ELC, essential light chain; S-1, subfragment-1.

agarose, FMC), and then fragments of approximately 210 and 180 bp were excised from the gel and purified. The purified product was ligated into the TA cloning vector (INVITROGEN) and used for the transformation of competent cells (INVαF'). Plasmid minipreps were performed and sequenced by the dideoxynucleotide chain termination method using Sequenase II (Amersham Pharmacia).

Construction of a cDNA Library—cDNA was synthesized from the total RNA using a SMARTTM cDNA Library Construction Kit (CLONTECH) according to the instruction manual, and then the cDNA library, which was inserted into the lamdaTriplEX2™ phage vector, was packaged with the Gigapack II Gold extract (STRATAGENE).

PCR Screening—To select myosin clones, PCR screening was carried out with the degenerate primer described above. Initially, approximately 3×10^5 plaques were plated onto thirty plates at a density of 10,000 plaques per plate. Phage particles from each plate were eluted into 5ml of SM buffer [0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.5), and 0.01% gelatin], and 1 μl of each separate phage suspension was used for the PCR reaction. Two of the thirty PCRs generated a product with the expected size of 210 bp. One of the phage suspensions from these positive plates was diluted for secondary screening and plated on ten plates at a density of 1,000 plaques per plate. PCRs were performed for each of the ten plates as described above and then the phage suspension from a positive plate was employed for the third screening. Thus, PCR screening was conducted sequentially as described above and a single positive clone was finally isolated. The plasmid (pTriplEX2™) was excised from the positive phage clone according to the instruction manual and the internal sequence was analyzed using a Dye-Deoxy™ terminator cycle sequencing kit with a DNA sequencer model 310 (Perkin Elmer).

Fusion Protein Expression—The fusion protein was constructed as follows. First, a 60 base pair fragment of cDNA encoding amino acid residues 2–14 was generated by PCR using a set of primers having the following sequences, as a sense primer: 5'-CGCGGATCCGCGGACGATCCGTACGC-3' containing *Bam*HI recognition sequences, and as an antisense primer: 5'-GGAATTCTACTTGAGCTCCTGA-3' containing *Eco*RI recognition sequences. The 60 bp PCR product was restricted, purified, and then ligated into the *Bam*HI–*Eco*RI site of the pGEX2 expression vector (Amersham Pharmacia). The plasmid was transformed into *Escherichia coli*, BL21 (DE3) strain. The fusion protein was expressed and then purified with Glutathione-Sepharose 4B (Amersham Pharmacia).

Production of Polyclonal Antibodies—An emulsion of 500 μg of the GST (glutathione-S-transferase)-fusion protein in Freund's complete adjuvant (1:1, v/v) was injected intradermally into a rabbit. For boost immunizations, an emulsion of 500 μg of the GST-fusion protein in Freund's incomplete adjuvant was injected two times at 2-week intervals, and the antiserum was collected regularly after the final immunization. The antiserum was affinity-purified over a synthetic peptide (ADDPYAGVS) corresponding to the N-terminal region of the mantle myosin.

Electrophoresis and Immunoblotting—Each tissue was homogenized in a solution containing 2% SDS, 20 mM Tris, 10% glycerol, and 0.1% 2-mercaptoethanol. Protein concentrations were measured by BCA assaying, and equal amounts of tissue extracts were separated by SDS-PAGE on a 12% gel (*12*). Proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk (w/v) in Tris-buffered saline containing 0.5 M NaCl and 0.05% Tween 20 (solution A) before the addition of primary antibodies diluted with solution A containing 1% BSA, and then the secondary antibodies, alkaline phosphatase–conjugated goat anti–rabbit IgG, diluted with solution A were added. Color development was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

RESULTS AND DISCUSSION

Isolation of a cDNA Encoding the Motor Domain of Myosin—To identify myosin II from scallop mantle tissue, the RT-PCR technique was used. Using degenerate primers derived from conserved sequences in the myosin head region (GESGAGKT and EAFGNAKT), 210 and 180 bp fragments were isolated. Sequence analysis revealed that the 180 bp fragment is 87% identical at the amino acid level to scallop (*Aequipecten irradians*) striated muscle myosin reported by Nyitray *et al.* (*13*). On the other hand, the 210 bp fragment has a longer structure than those of scallop striated and catch muscle myosins (Fig. 1). If this nucleotide sequence is derived from that of myosin, the region corresponds to a loop 1 structure between 27 and 50 kDa. It has been implied that the structural variations in loop 1 influence the motor function of the myosin molecule (*14–16*). To confirm the 210 bp fragment is myosin, screening of a longer clone from the scallop mantle cDNA library was carried out as described under "MATERIALS AND METHODS," and the isolated cDNA clone was subjected to DNA sequencing.

Primary Structure of the Mantle Myosin—The determined nucleotide sequence and deduced amino acid sequence are shown in Fig. 2. It comprises 3,547 bp with a part of the 5'-noncoding sequence and has an open reading frame of 3,463 bp which encodes 1,154 amino acids. The deduced amino acid sequence revealed that this myosin has characteristic ATP binding (GESGAGKT) and actin binding (VRCIIPN) sites, and shares many conserved residues with other myosins.

Comparison of the Mantle Myosin with Scallop Muscle Myosin—Alignment of the motor domain of the mantle myosin with those of other myosins is shown in Fig. 3. Comparison of the mantle myosin with scallop muscle myosin revealed that highly conserved regions include the P-loop (residues 201–208), switch I (residues 272–278), and switch II (residues 493–498), which form part of the active site of myosin, the reactive SH region (residues 727–737), and the actin binding site (residues 686–708). On the other hand, the least conserved regions are observed in highly variable regions of myosins, protease-sensitive regions and the N-terminal region (residues 1–75). Scallop striated muscle subfragment-1 (S-1) has three tryptic sites, the 25K–50K junction (loop 1), 50K–20K junction (loop 2), and 65K–30K junction (residues 559–577, scallop striated muscle numbering), which is characteristic in scallop striated muscle S-1. The sequence of the mantle myosin in loop 1, loop 2, and the 65K–30K junction is greatly different from the corresponding regions of scallop muscle myosin. Loop 1 and loop 2 of the mantle myosin have longer structures than those of scallop muscle myosin.

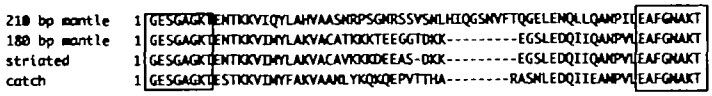

Fig. 1. **Sequence alignment of amplified fragments.** The deduced amino acid sequences of amplified fragments (210 and 180 bp) with a set of degenerate primers are compared with scallop striated and catch muscle myosins (*7*, *10*). The amino acid sequences corresponding to a set of degenerate primers are boxed. Bars indicate gapped amino acids.

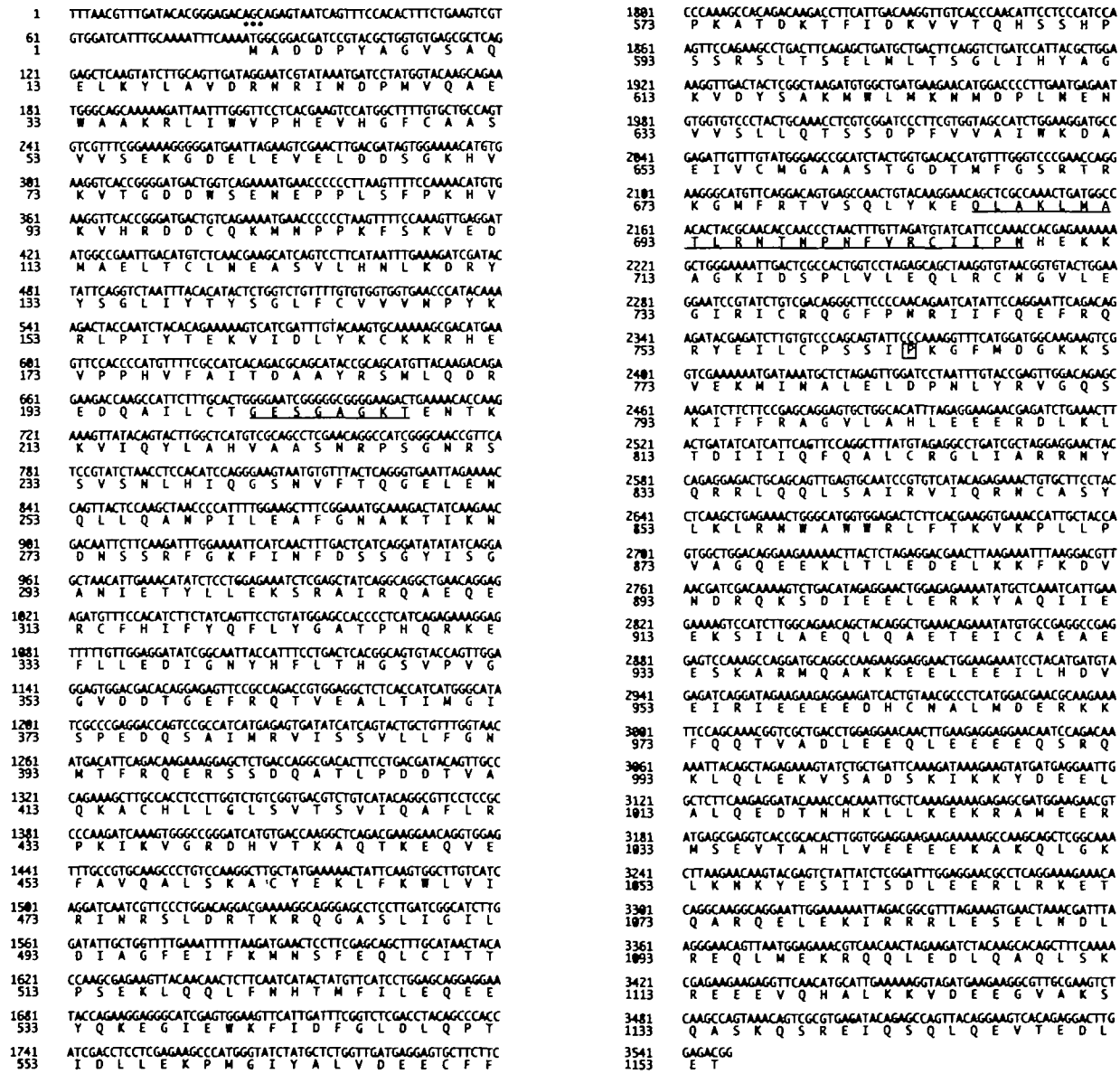

Fig. 2. **Nucleotide and deduced amino acid sequences of mantle myosin.** The start codons are shown by asterisks. Solid lines below amino acid sequences represent the sequences of ATP (thick line) and putative actin (thin line) binding sites. Pro_{872}, which marks the beginning of the head/rod junction, is boxed.

Comparison of the Mantle Myosin with Other Myosins—Comparison with various kinds of myosins revealed that scallop mantle myosin is similar to vertebrate non muscle and smooth muscle, and invertebrate non muscle myosins rather than scallop striated and catch muscle myosins. The sequence exhibits 65% identity with *Drosophila* non muscle myosin (*17*), 60% with chicken gizzard smooth muscle myosin (*18*), 59% with human nonmuscle type-A myosin (*19*), 41% with chicken skeletal muscle myosin (*20*) and 43% with scallop striated muscle myosin (*13*) at the amino acid level (Fig. 3). A phylogenetic tree generated from myosin head domain sequences using the ClustalW program showed that the myosins are divided into three major groups (Fig. 4): a striated muscle myosin group, a smooth/non muscle myosin group, and a lower eukaryote myosin group. Scallop striated muscle myosin is classified in the

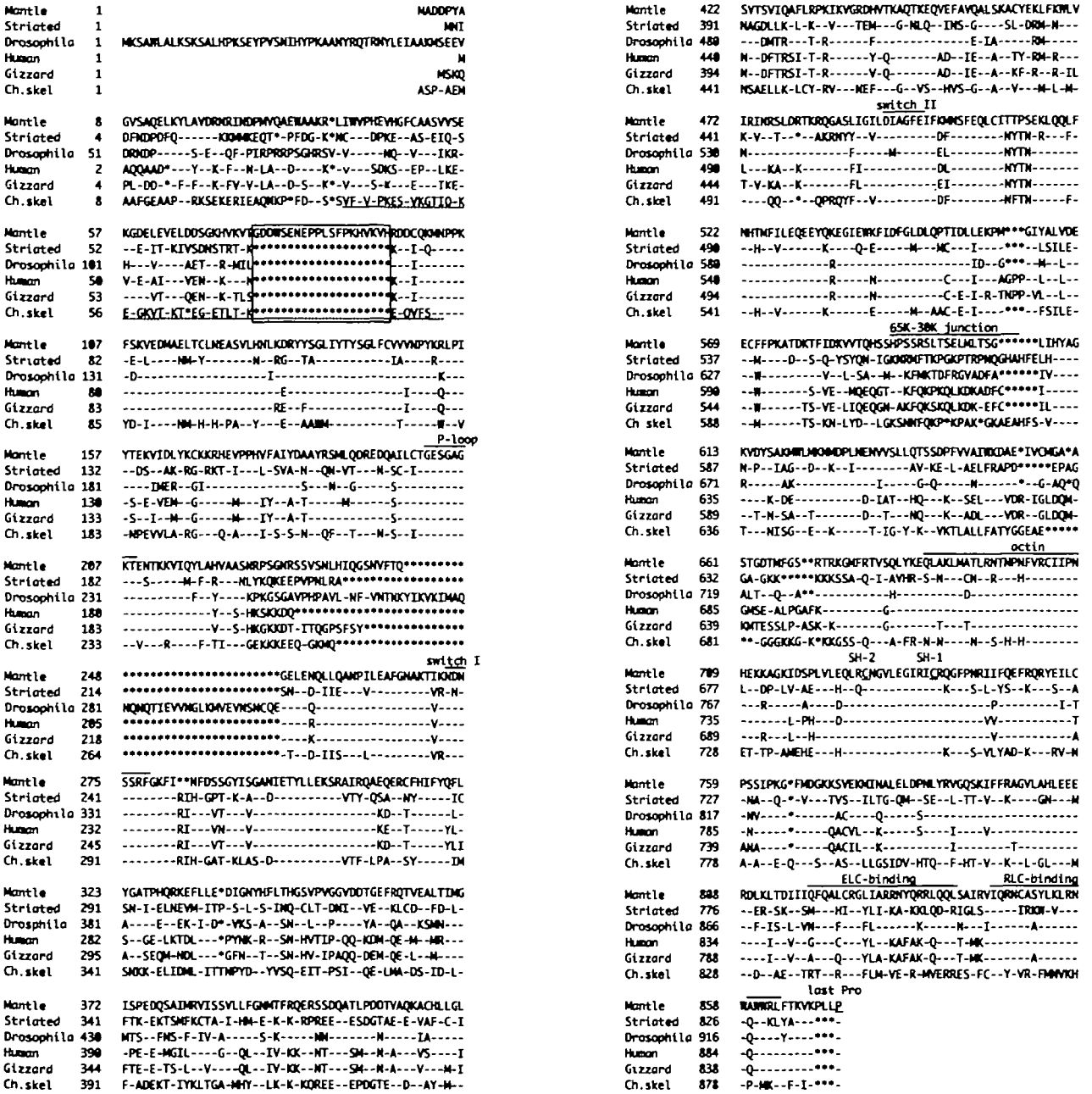

Fig. 3. **Similarities between mantle myosin and other myosin heavy chains.** The deduced amino acid sequence of the motor domain of the mantle myosin is compared with the head sequences of scallop striated muscle myosin, *Drosophila* non muscle myosin, chicken gizzard smooth muscle myosin, human nonmuscle type-A myosin, and chicken skeletal muscle myosin. Mantle, scallop mantle myosin; Striated, scallop (*Argopecten irradians*) striated muscle myosin; Drosophila, *Drosophila* non muscle myosin; Gizzard, chicken gizzard smooth muscle myosin; Human, human nonmuscle type-A myosin; Ch.skel, chicken skeletal muscle myosin. Dashes and asterisks indicate identical amino acids and gapped amino acids, respectively. The 65K–30K junction, ATP-binding (P-loop, switch I, and switch II), actin binding, essential light chain (ELC)-binding, and RLC-binding sites are indicated above the amino acid sequences. SH-1, SH-2 and the last Pro are also underlined. The insertion within the SH3 domain, which is shown by a dotted line, is boxed.

striated muscle myosin group, whereas the mantle myosin is classified in the smooth/nonmuscle myosin group. This was also supported by the following segment comparison. Table I shows simple segment comparison of the motor domain of the mantle myosin with those of other myosins in the three regions of the 25K domain (residues 1–225), 50K domain (residues 248–646), and 20K domain (residues 670–868). The 25K–50K junction and 50K–20K junction were omitted from the comparison. The 50K and 20K domains of the mantle myosin are more homologous to those of smooth/nonmuscle myosins than the 25K domain, because the 25K domain includes a long variable region at its N-terminus. The segment comparison revealed that the sequences clearly fall into two groups; striated and smooth/nonmuscle myosins. It is clear that the three domains of the mantle myosin are more similar to the corresponding

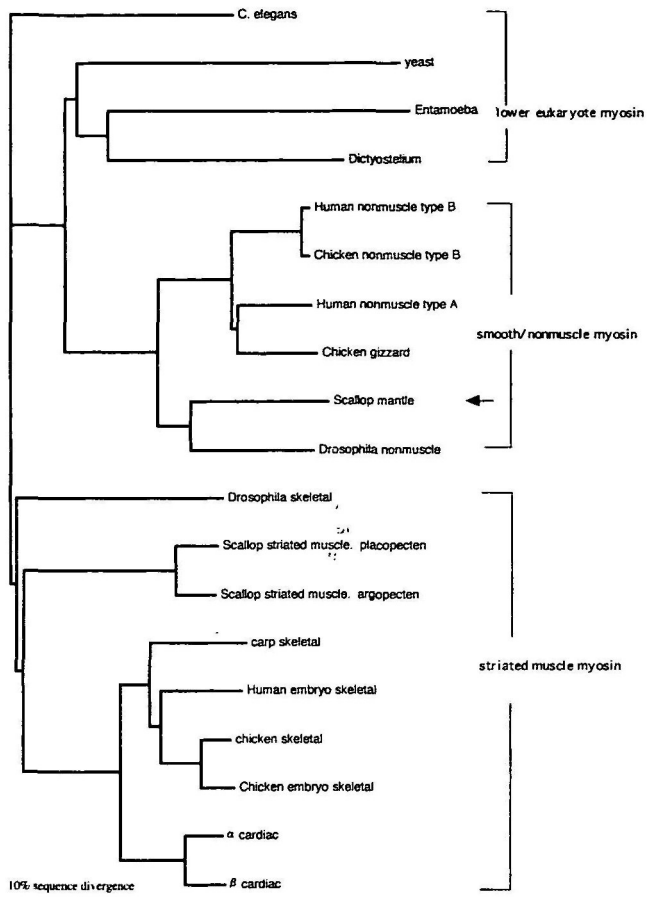

Fig. 4. **Phylogenetic tree of myosin II based on the sequences of the motor domain.** The sequences from the amino terminus to the end of the motor domain (last Pro) of various kinds of myosin II were taken. The sequences were aligned and a bootstrap tree file was created, and then a phylogram tree was drawn with the TreeView program. The mantle myosins is indicated by an arrowhead. Accession numbers: *C. elegans*, P02566; yeast, P08964; *Entamoeba*, L03534; *Dictyostelium*, P08799; human nonmuscle type B, M69181; human nonmuscle type A, M69180; chicken nonmuscle type B, M93676; chicken gizzard smooth muscle, P10587; *Drosophila* nonmuscle, M35012; *Drosophila* skeletal muscle, M61229; scallop striated muscle (*Placopecten magellnicuss*), U59294; scallop striated muscle (*Argopecten irradians*), U09782; carp striated muscle, D89992; human embryonic skeletal muscle, P11055; chicken skeletal muscle, P13538; chicken embryonic skeletal muscle, P02565; rat β-cardiac muscle, P02563; rat α-cardiac muscle, P02563.

TABLE I. **Segmental comparison of the motor domain of the mantle myosin with those of other myosins.**

	S-1	25K	50K	20K
Ch. gizzard smooth muscle	59	54	64	80
Dm. non muscle	65	53	72	85
Hu. non muscle	59	53	62	81
Sc. striated	44	41	49	53
Dm. striated	40	36	45	49
Ch. skeletal	41	35	49	50

The sequence of each domain of the mantle myosin was compared with the corresponding region of other myosins. The percentage sequence identity was calculated with the DNASIS program (HITACHI software). 25K, 25K domain (residues 1–225); 50K, 50K domain (residues 248–584); 20K, 20K domain (residues 646–868). The abbreviation used are: Ch, chicken; Dm, *Drosophila*; Hu, human; Sc, scallop.

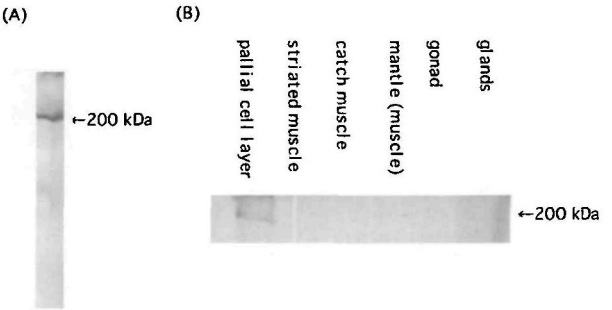

Fig. 5. **Detection of mantle myosin in scallop tissues by Western blotting.** Scallop tissues were extracted with 2% SDS, 20 mM Tris, 10% glycerol, 0.1% 2-mercaptoethanol. (A) The mantle extract was immunoblotted with a polyclonal antibody against the N-terminal domain of mantle myosin as described under "MATERIALS AND METHODS." (B) Equal amounts of total extracts of different tissues were separated by SDS-PAGE and then immunoblotted. The tissues are indicated at the tops of the lanes. A molecular weight standard (200 kDa) is indicated by arrowheads.

regions of smooth/nonmuscle myosins than striated muscle myosins. This led to the conclusion that the mantle myosin belongs to the smooth/nonmuscle type myosin group, different from scallop muscle myosin.

The scallop mantle myosin shares a 20 amino acid insert in the N-terminal region, which has been termed the myosin SH3 domain (*21*). Although the function of the SH3 domain is not clear, it may not be essential for motility in that it is missing in several unconventional myosins. The following two functions have been suggested for the N-terminal domain including the SH3 domain. (i) The tail region of the myosin head, which is pictured as a "lever arm" in the crystal structure, rotates upon ADP release accompanying ATP hydrolysis (*22*). Dominguez *et al.* suggested that the N-terminal domain of myosin may limit the potential swing of the lever arm during the cross-bridge cycle and alter the step size of myosin (*23*). (ii) The two heads of chicken gizzard heavy meromyosin in a rigor complex with F-actin could be cross-linked by a zero-length cross linker (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide) (*24*). The cross-linking occurs between two residues, Lys-65 of one head and Glu-168 of the other, suggesting that the N-terminal region is involved in the interaction between the two heads bound to F-actin. These suggestions indicate the possibility that the insertion in the 27 kDa domain could influence the motor function.

Tissue Distribution of Scallop Mantle Myosin—To investigate the tissue distribution of the mantle myosin, antiserum was raised against a bacterially expressed myosin fragment (amino acids 2–14) fused to GST, and then affinity purified over a synthetic peptide (ADDPYAGVS), as described under "MATERIALS AND METHODS." This antibody recognized a 200 kDa band for the mantle extract (Fig. 5A), which corresponds to the molecular weight of the myosin heavy chain. A tissue distribution study with this antibody demonstrated that this myosin was specifically expressed in the mantle pallial cell layer (Fig. 5B). Barely detectable levels were observed in the striated muscle, catch muscle, mantle (muscle portion), glands, and gonads. The fact that the mantle myosin is specifically expressed in the pallial

cell layer consisting of nonmuscle cells supports that the mantle myosin is a nonmuscle type myosin.

In conclusion, we isolated a cDNA fragment encoding the motor domain of myosin from the mantle tissue including the pallial cell layer. This myosin was specifically expressed in the mantle pallial cell layer. This is the first report of a nonmuscle type myosin being found in scallop.

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