

Catchin, a Novel Protein in Molluscan Catch Muscles, is Produced by Alternative Splicing from the Myosin Heavy Chain Gene

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Molluscan catch muscles contain polypeptides of 110–120 kDa in size which have the same partial amino acid sequences as those of the myosin heavy chain (MHC). Here we provide evidence that these polypeptides are major components only of the catch-type muscles (their estimated molar ratio to MHC is approximately 1:1) and they are alternative products of the MHC gene. Northern blot analysis of total RNA from *Mytilus galloprovincialis* catch muscles was carried out with fragments from the 3'-end of the MHC cDNA as probes. We detected two bands of 6.5 kb and 3.5 kb. The former corresponds to the MHC mRNA, and the latter is an mRNA coding for catchin, a novel myosin rod-like protein. By using a 5'-rapid amplification of cDNA ends (RACE) PCR method, the full-length cDNA of *Mytilus* catchin was cloned. It codes for a protein with a unique N-terminal domain of 156 residues (rich in serine, threonine, and proline), which includes a phosphorylatable peptide sequence. The rest of the sequence is identical with the C-terminal 830 residues of the MHC. We also analyzed *Mytilus* and scallop (*Argopecten irradians*) genomic DNAs and found that the 5'-end of the cDNA sequence was located in a large intron of the MHC gene in both species. Since catchin is abundantly expressed only in catch muscles and it is phosphorylatable, we suggest that it may play an important role in the catch contraction of molluscan smooth muscles.

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Introduction

Molluscan smooth muscles have large thick filaments that consist of a core of paramyosin covered with a layer of myosin (Szent-Györgyi *et al.*, 1971; Cohen, 1982). Some of these muscles have a unique physiological function known as catch. After the active contraction, these muscles can enter the catch state, under which they maintain high tension without much expenditure of energy at a rest-

ing level of intracellular Ca^{2+} concentration. A similar state exists in vertebrate tonic smooth muscles termed "latch". In both cases the cross-bridges somehow remain attached to the actin filament and cycle at a very slow rate (for reviews, see Twarog, 1979; Chantler, 1991). The most extensively studied catch muscle is the anterior byssus retractor muscle (ABRM) of the common mussel *Mytilus edulis*. As in all molluscan muscles Ca^{2+} induces active contraction by binding directly to myosin (Kendrick-Jones *et al.*, 1970). In ABRMs, catch subsequently occurs after decrease of Ca^{2+} to resting level (Astumi & Sugi, 1976; Ishii *et al.*, 1989). Catch contraction is regulated by phosphorylation. Release of catch is mediated by a cAMP-dependent protein kinase (Cole & Twarog, 1972). It has been recently shown that the major target of the kinase is twitchin/mini-titin, a giant protein associated with thick filaments (Siegmán *et al.*, 1997, 1998). Interestingly, twitchin phosphorylation also effects force output of *Mytilus*

Abbreviations used: ABRM, anterior byssus retractor muscle; PBRM, posterior byssus retractor muscle; PAM, posterior adductor muscle; PRM, pedal retractor muscle; MHC, myosin heavy chain; RLC, regulatory light chain; EGTA, ethyleneglycol-bis-(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; DIG, digoxigenine; PAGE, polyacrylamide gel electrophoresis; RACE, rapid amplification of cDNA ends.

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ABRM during calcium-mediated submaximum contractions (Butler *et al.*, 1998). The catch state requires the twitchin to be dephosphorylated, probably through the action of a calcium-activated phosphatase (Castellani & Cohen, 1992). Other thick filament components were also shown to be phosphorylatable *in vitro* by an endogenous kinase - paramyosin (Cooley *et al.*, 1979; Achazi, 1979; Cohen, 1982), myosin heavy chain (MHC) (Castellani & Cohen, 1987) and myosin regulatory light chain (RLC) (Sohma *et al.*, 1985); their potential role in the regulation of the catch state is more controversial. The mechanism of catch contraction has been much discussed. It was proposed that the slow cycling cross-bridges during catch state are somehow related to the unusual organization of the thick filaments where critical changes of protein-protein interactions are brought about by phosphorylation (Cohen, 1982).

Thick filaments of catch muscles compared with those of non-catch muscles, besides their much higher paramyosin content, possess different myosin isoforms with different enzymatic properties. In particular, catch muscle myosins have considerably lower steady-state ATPase activity than myosins from phasic muscles. Catch and striated muscle specific RLC isoforms were identified in *Patinopecten yessoensis* and *Placopecten magellanicus* (Kondo & Morita, 1981; Perreault-Micale *et al.*, 1996a). However, it was shown that they are not responsible for the muscle-specific differences in enzymatic activities. Instead, those differences are attributable to MHC isoforms (Perreault-Micale *et al.*, 1996b). In the Pectinidae family, a single MHC gene generates catch and striated muscle specific MHC isoforms by alternative RNA splicing (Nyitray *et al.*, 1994; Perreault-Micale *et al.*, 1996b). Among the three alternative exons in the motor domain of MHC, sequence variations within the exon forming the flexible surface loop I at the ATP binding pocket modulate ADP affinity, ATPase activity and *in vitro* motility of scallop catch myosin (Kurzawa-Goertz *et al.*, 1998). Much less is known about the role of the two alternative sequences in the middle of the coiled-coil rod region and at the C-terminal non-helical tailpiece (Nyitray *et al.*, 1994).

It has previously been noted that the crude myosin or thick filament preparations from *Mytilus* ABRM contain considerable amounts of an additional protein whose apparent molecular mass is around 110-140 kDa (Castellani *et al.*, 1988; Yamada *et al.*, 1989). Originally it was thought to be a degradation product of the myosin rod, however later studies suggested that the myosin rod-like protein is produced in the muscle cells (Yamada *et al.*, 1997). Here we provide evidence that the ABRM myosin rod-like protein, named catchin, is an alternative product of the MHC gene. Since it is abundantly expressed only in molluscan catch muscles, we suggest that it may contribute to the force-maintaining structures during the catch state.

Preliminary results of this work have been published elsewhere (Yamada *et al.*, 1999).

Results

A new protein component in bivalve muscles

Recently, two new polypeptides (120 kDa and 112 kDa) were identified in *Mytilus* catch muscles as myosin rod-like proteins. Their partial amino acid sequences were found to be identical with those of the MHC from the same muscle (Yamada *et al.*, 1997). In this report we analyzed protein compositions of various muscles from *Mytilus galloprovincialis*, *Crassostrea* sp. and several species of the Pectinidae family (*P. yessoensis*, *Argopecten irradians*, *P. magellanicus*, *Pecten maximus*) by SDS-polyacrylamide gel electrophoresis (PAGE), to see whether they contain similar components (Figure 1). Anterior byssus retractor muscle (ABRM), posterior byssus retractor muscle (PBRM), and posterior adductor muscle (PAM) of *Mytilus* contained considerable amount of the rod-like proteins (Figure 1, lanes 1-3). The staining intensity ratios of the two new bands to MHC and paramyosin were 1:2 and 1:5, respectively. The molar ratio of the new protein to MHC is approximately 1:1, assuming that the specific staining is similar between them. In contrast, pedal retractor muscle (PRM), which is not a catch muscle (Ishii & Takahashi, 1981), contained only a small amount of the rod-like proteins (Figure 1, lane 4; the staining intensity ratio to MHC was 1:25). The rod-like protein was found to be heat-stable, and could easily be purified by heat treatment (compare Figure 1, lanes 5 and 6). Therefore, we identified the corresponding protein in various muscles by this simple method (Figure 1, lanes 8-15). In oyster, a heat-stable component of about 120 kDa was found abundantly in the white adductor catch muscle, but only in small amount in the translucent non-catch adductor muscle (Figure 1, lanes 12-15). In the catch adductor muscle of *Patinopecten*, there was a component of about 120 kDa and it was heat stable (Figure 1, lanes 8 and 10). In contrast, there was no such a component in the striated adductor muscle (Figure 1, lanes 9 and 11). The same results were obtained with the three other scallop species (results not shown). Thus, the heat-stable proteins are abundant only in catch muscles, and the name "catchin" was coined to them.

Cloning of the rod portion of MHC from *Mytilus* catch muscles

It was suggested that the 120 kDa and the 112 kDa myosin rod-like polypeptides of *Mytilus* catch muscles had been produced in the muscle cells, rather than by proteolytic degradation of MHC during the isolation procedure after the muscles were dissected from the animals (Yamada *et al.*, 1997). If catchin is a genuine muscle protein,

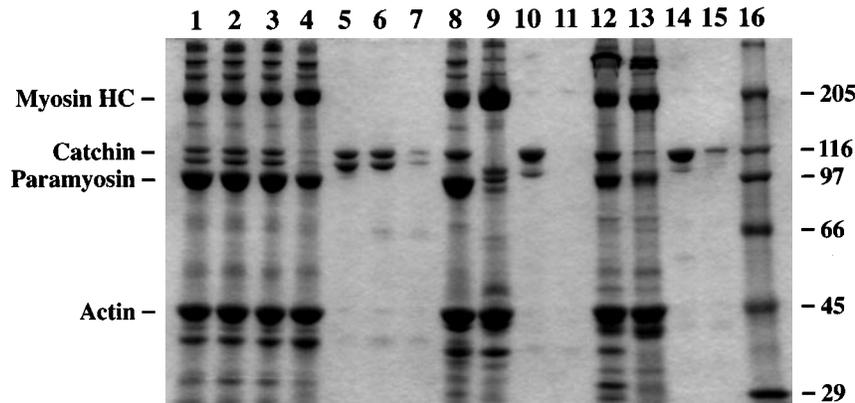


Figure 1. Protein compositions of muscles from different bivalves (*Mytilus*, *Patinopecten* and *Crassostrea*). Protein compositions of whole muscle homogenates (lanes 1-4, 8, 9, 12 and 13) and their heat-stable fractions (lanes 6, 7, 10, 11, 14 and 15) are shown on SDS-7.5% PAGE. Samples were prepared from ABRM (lanes 1 and 6), PBRM (lane 2), PAM (lane 3) and PRM (lanes 4 and 7) of *M. galloprovincialis*, catch adductor (lanes 8 and 10) and striated adductor (lanes 9 and 11) of *P. yessoensis*, and catch adductor (lanes 12 and 14) and translucent

cross-striated adductor (lanes 13 and 15) of a *Crassostrea* sp. Catchin prepared from *M. galloprovincialis* catch muscles according to Yamada *et al.* (1997) was also analyzed (lane 5). Note that the heat-stable components of about 120 kDa in size (catchin) are abundant only in catch muscles. Lane 16 shows a molecular mass protein markers (Sigma, SDS-6H) with molecular masses (kDa) on the right.

the cells must contain an mRNA which is considerably smaller than that of the MHC. Moreover, since the catchin polypeptides have, at least partially, identical amino acid sequences to that of the MHC rod, it is likely that their mRNA sequence is also partially identical and that catchin is produced by alternative RNA splicing from the MHC gene. In order to confirm this possibility, Northern blot experiments and genomic sequence analysis (see below) were carried out.

We first cloned and sequenced cDNA fragments coding for the MHC from *M. galloprovincialis* catch muscles as outlined in Materials and Methods (Figure 2(a); GenBank accession no. AJ249991). In Figure 3, the deduced amino acid sequence of myosin rod is shown aligned with the sequences of myosin rods from *M. galloprovincialis* PRM (see below) and from *A. irradians* catch adductor muscle (Nyitray *et al.*, 1991, 1994). Peptide sequences of *Mytilus* rod which were previously reported are underlined (sequences A and B were described by Yamada *et al.*, 1997; sequence C was by Castellani *et al.*, 1988). The fourth residue of sequence A is Tyr instead of Val as reported previously. This could be explained by a modification of the Tyr side-chain during the *o*-iodosobenzoic acid treatment and the erroneous identification of the modified residue as Val (Yamada *et al.*, 1997). The C-terminal residue in sequence C is an Asp residue instead of a Glu residue. This phosphorylated peptide, however, was isolated from ABRM of *M. edulis*. Comparison of the sequence of a 1 kb fragment from *M. edulis* MHC cDNA (L.N., unpublished results; Genbank accession no. U40036) with the rod sequence reported here, shows the same Glu-Asp change at the C-terminal residue. The rest of the differences are at either silent codon positions or within the 3'-non-translated region (99% identity in the coding and 97.6% in the non-coding region). The very high degree of conservation reflects the fact that *M. galloprovincialis* and *M. edulis* belong to one species complex.

Northern blotting analysis of four muscles from *M. galloprovincialis*

Since the 120 kDa and the 112 kDa polypeptides have partially identical amino acid sequences to those of the MHC rod, putative mRNAs for these polypeptides would hybridize with probes for the myosin rod cDNA. Two probes were prepared (see Figure 2(c)) and hybridized to total RNAs from four muscles of *M. galloprovincialis* (Figure 4). Hybridization of ABRM, PBRM, and PAM RNA with probe 1 shows two bands of 6.5 kb (indicated by an arrowhead) and 3.5 kb (indicated by a tandem arrowhead). The 6.5 kb band is the mRNA of MHC. The presence of a 3.5 kb band indicates that

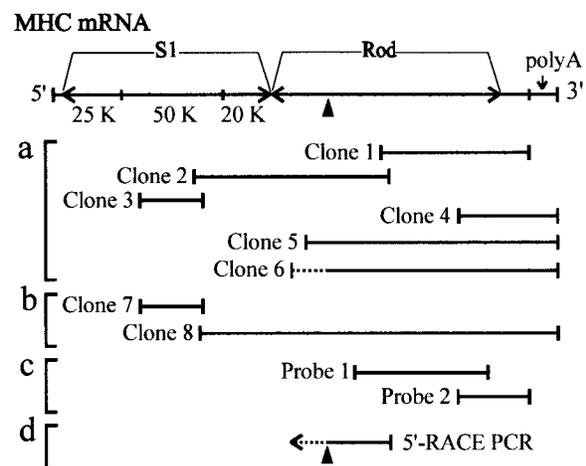


Figure 2. Schematic view of the MHC and positions of the various cDNA fragments and probes used in this study. Cloned and sequenced cDNA fragments of the MHC of (a) catch muscles (ABRM, PBRM and PAM) and (b) of PRM from *M. galloprovincialis*. (c) Probes for Northern blotting analysis. (d) cDNA fragments of catchin obtained by 5'-RACE PCR experiments. Dotted lines show the N-terminal unique part of catchin. See Materials and Methods for details.

a, 1 PLLNIARAEEMKKKIEEMGKLRDLAKCERLKKLELVQNVTLLEQKNDLYLQLQTEQDAVADLEERVEKLVTKADFS
b, -M-----E---Q--L---K-ME--I--L-KI-----IK--E-----M-----S---N-V---Q--Q-I---E---
c, ---S---Q-----EQLKQ-D-MK-----T--I-----E-----F-----LE-SMG-Q-----IM-----

81 QIKEMEERLLDEEDAAAELEVIKRMEGENDELKKDIEDLENSLAKSEQEKTTKDNQIKTLQDEMAQQDDIISKLNDRDKK
 -M-----S---GL-K-----S-M-R-----TT---A-D-H-----G-----EQ---GKE--
 ---L-----D-G-K---AD-AN---G---T-Q-A-D-AH---S---G-IS---EH-G---KE--

161 GMDEDHKRTLQKEEDKVNHLKSLKQKLESTLDELEDGLEREKKVGRGDVEKAKRKIEQDLKATQEAVEDLERVKRGLN
 NLE-VN-K--AD-----N-I-A-----M-N-----S---V-----V-E-G---EM-
 ALE-AN-K-S-S-A---C---N---A---QA-----N-----V-----S---N-----E--

↓

241 ETNRKKDAENAALSSRLEDDQSLIAQLQRKIKELLARIEELEELEAERAARTKVQKQRAEIARELEDLSERLDEAGGAT
 -AV-R--V-LNS-H--I--CEGVN-NQS-----QQI-----A-E---N-L---DE--SQ-E-Q---
 -NV-R-E--ISS-N-K---E-N-VS-----Q-----N--A-E-----LN---E-G-----

321 SAQIELNKKREQELLKLRDLEETTLQHEAQVSSLRKKQDAANEMADQIDQLQKAKSKTEKERQQFKSECDDLQSQLQH
 Q--MD-----H--MR---EM--M---S---AQF-----ESN-----N-L--RL---KKDL-R-L--V---S-
 -----A---I-----AS-----I-A---H-----V---V---S---K--LR--VE---A-I--

401 ISKNKGVSEKMAKSLENTIAELQHKCDDSNRNVNDLNTQKAKMQAENANI IQQLEDVEHQCSAITKERNAMQSQLDEMRA
 SM--R--D-V--QY-SQ-S-ITY-VEETQ-TIVEI-S--S-L-S-VTE-NR---A--TIGSL--DKS-LT---E-AKR
 -----C---VM-QF-SQMSD-NARLE--Q-SI-E-QS--SRL---SDLTR---A--RV-VLS--KSQLS---EDA-R

A

481 ALEETRARQKLGDIRNLSDDLGLRESNEEEQEAELQRLLSKANNEAQQWRVVKYESEGANKAELEEARLKLOAKL
 S-----M---F-SEV---A-V-SV---L-----S-SD---Q---K-----S---T---A---D---SK---S---
 S-----S---NEV--MHA-M-AI--QL-----S-SDV--Q-----I---S-F-----RT---DQK---LG--

B

561 QEAEQNAEAAANAKVSSLEKAKNRLTGELEDLGDVERANANANSLEKKQRAFDKTIQEWQAKVTDLQSELENAQKEARSY
 A---A-----A-G-----Q---D--L-E---S-V-SST-----Q--R-----TL--KE--T-IDT-HT---G-
 S---TT-----C-A-----S--QQ---MS-E-D---SV-QM-----TA-----NS-----S---S-G-

641 SAELFRCKAQYEEESQDSVEALRRENKNLAEIHELTEQLSEGGRSVHEVEKAKRRLEMEKEELQAALAEAEALEQEEAK
 -----V-----TS-TI-----D--R--SD---D---S--I-----
 ---Y-I--SI--Y--IG-----D--D--D-----T--LD--R-----G-----

721 VMRGQLEISNVRSEIERLQEKDEFENTRRNHQALDSMQASLEAEAKGKAEAMRIKKKLEQDINELEIALDSSNRAKA
 -V-S--M-TI-T-D--IH-E-----E--I--D-----H--R-----M--T-----
 --A---AT--N--DK-I--E--D-----E-----D-----V---A---G--

801 ELEKNIKRYQQQVSEMQRQIEEQRQREEVRESYNMAERRCNMLSGEVEELRTALEQAERARKGAENELFEANDRVNELS
 -A---L---IV-IQ-LTLKL---T-A-D-S---V-I---L-VS--D-V-S---S---T---S---Y-SQ--I---A
 -M--TV-----IR--TS-----D-A-----TLM-----A-----ASD---AD-----T

881 AEVQSAQSSKRKLEGDIQAMQSDLEMNNEVRNADERARRANDDSARLADELRSQEHSQQIEKFRKSLESQVKDLQVRL
 -Q-S---AQ---V--M---SA---LQ--IKA--D--K--VM-TT--S---A---S---M--T--ITI-E-H--V
 SQ-S-V-GQ-----N---T---HG-LKG---CKK-MA-A-----A-D--N-V--V--N-----EF-I--

961 EEAEAQALKGGKMKIAKLEQVRVRELEGELDSEQRRAETQKHMRAKDRRLKEIAFQADEDRKNQENLNEMIEKLNKLT
 D---SGV--R-V-----QD--S--N-----N-----M--L--C---G--R-TD--D--QS-V--
 D---SS-----Q--S--H--A--N-----N-----L-----R-Q-L-D--A-I--

C

↓

1041 YKRQVEEAEIEAAILAKYRKVQOELEDAEERADSAEGSLQKLRANKRSSSVVTRTSYTTSTLD
 F-----A-----E-S-A--T--T---M-S---IKSV-VSNMSSQ
 F-----A-H--E-----T-DST--F--S-----Q-S-VSV-ASNAAHVAHHHVE

Figure 3 (legend opposite)

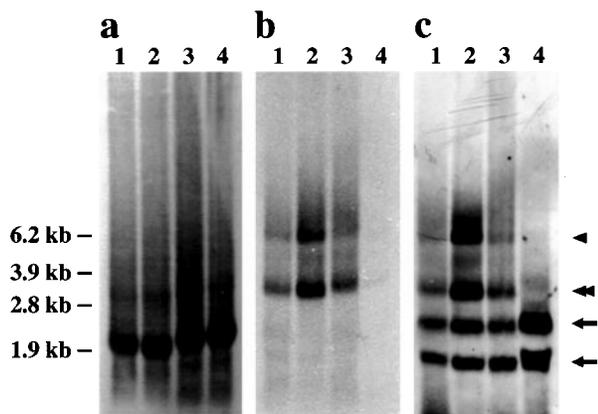


Figure 4. Northern blotting analyses of *Mytilus* muscles. Total RNAs prepared from (1) ABRM, (2) PBRM, (3) PAM and (4) PRM were electrophoresed and transferred to a nylon membrane. The membrane was stained with either (a) ethidium bromide or (b) probe 1. In addition to the bands of mRNA for MHC of 6.5 kb (arrowhead), bands of 3.5 kb (tandem arrowhead) were detected in RNAs from ABRM, PBRM and PAM. (c) When we used a probe for *Mytilus* tropomyosin together with probe 1, additional bands of tropomyosin mRNAs (arrows) were detected in all RNA preparations.

a truncated mRNA, presumably coding for catchin, was also transcribed in these muscles. It is notable that the 3.5 kb RNA is abundant only in catch muscles (ABRM, PBRM and PAM), similarly to the two polypeptides of the rod-like protein (Figure 1). When the Northern blotting experiment was carried out with a mixture of probes for myosin rod and for tropomyosin (obtained from a *M. edulis* tropomyosin clone, L.N., unpublished results, GenBank accession no. U40035), two additional bands of about 2.3 kb and 1.3 kb were detected in all muscles (Figure 4(c)). Thus the fact that the 6.5 kb and 3.5 kb bands were rather faint in the PRM sample (Figure 4(b), lane 4) is explained by the much lower expression level of these mRNAs in PRM and not by degradation of RNA. The same results were obtained by using probe 2 (data not shown). These results suggest that the sequence of MHC in PRM is considerably different from that of the MHC of catch muscles. Indeed, sequenced cDNA fragments of MHC from PRM (clones 7 and 8) are different from that of the catch muscle MHC (identity 75%; GenBank accession no. AJ249992).

The deduced partial amino-acid sequence of PRM MHC shows 78% identity (84% similarity) with that of the catch muscle MHC (Figure 3).

N-terminal unique domain of catchin

In order to determine the N-terminal region of catchin polypeptides, we performed 5'-rapid amplification of cDNA ends (RACE) PCR experiments using specific primers based on the MHC cDNA sequence coding for sequence A (Figure 3(a)) which is common in catchin and MHC. Four independent cDNA clones from the 5'-end of the 3.5 kb RNA were sequenced (Figure 2(d); GenBank accession no. AJ249993). The sequence obtained encodes a 156 residues stretch of peptide, representing a unique N-terminal domain of catchin (Figure 5(a)). The corresponding sequence of *Argopecten*, which was obtained by analysis of the MHC gene (see below), is also shown (Figure 5(b)). The two homologous sequences show rather low similarity (42% similarity; 33% identity). The sequence in the second half of the unique domain is more conservative than in the first half (68% similarity, 53% identity), suggesting that this region is functionally more important. The N-terminal domain of catchin does not seem to form a coiled-coil structure (as analyzed by COILS; Lupas *et al.*, 1991) and it does not show strong propensity for any secondary structure element (results not shown). It is indeed a unique sequence: No similarity was found with any sequences in the databases. Within the unique N-terminal domain, we identified one of the phosphorylatable peptides (underlined in Figure 5) reported by Castellani *et al.* (1988). They assumed this peptide was in the C-terminal part of the MHC. However, our finding indicates it was actually in the N-terminal part of catchin.

Although there are two different polypeptides of 120 kDa and 112 kDa in *Mytilus* catch muscles, only a single sequence was obtained among the 5'-RACE products. Since there is an internal Met residue in the N-terminal domain of the *Mytilus* sequence (Met67), it is possible that translation also begins at this internal ATG triplet resulting in the 112 kDa polypeptide. In contrast, there is no second Met residue in the scallop sequence, in accord with the result that only a single main band was found in the heat-stable fraction of the scallop catch muscles (Figure 1, lane 10).

To get information from the C-terminal region of the catchin sequence, a 3'-RACE PCR experiment was performed using a catchin-specific 5'-primer

Figure 3. Amino acid sequences of the rod region of MHCs. Deduced amino acid sequences of the MHCs from the highly conserved Pro residue, marking the head rod junction, of (a) catch muscles and (b) of PRM from *M. galloprovincialis*, and (c) the catch adductor muscle of *A. irradians* (Nyitray *et al.*, 1991, 1994) are shown. Dash indicates an identical amino acid residue to that of the *Mytilus* catch muscle MHC. Amino acid sequences reported previously are underlined (A-C). Sequences A and B were reported by Yamada *et al.* (1997), and sequence C by Castellani *et al.* (1988). The boundary between the MHC-specific sequence and the sequence common to MHC and catchin is indicated by an arrow. A phosphorylatable Ser residue is indicated by an arrowhead (Castellani *et al.*, 1988).

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a, 1 *MSDTKITVTSTISTEEKPVVVKEESATVERIPTDIISEPPAPVAAKVTTVTEKSTSKASSQLSPKMSSTTTRTTKT
b,  ---GEQLKVEFS-SVETSQKDESPQPPPLDT-S-QAA-VIEVKSQV-A-SNPAPKSEI-PAETRKTTTTTTSSKKS--S
      *
      *
      ▼
81 SVGRSSDYESTIGQLTRDYRGTSPAVLEGIASHPIIYSKTFDSKIGRQKLSARSKILRDTSDLAIVAPGLKNLLE
   -TS---G-SDYL-DIS-----V-----I-S--T--V-F-RSYQYEPSTR-----RV--EA---SLTS---G---

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Figure 5. Amino acid sequences of the N-terminal unique domain of (a) *Mytilus* and (b) *Argopecten* catchin. The N-terminal sequences of *M. galloprovincialis* and *A. irradians* catchin were obtained from 5'-RACE-PCR products and the MHC gene, respectively. In the *Mytilus* sequence, one of the phosphorylatable polypeptide sequences reported by Castellani *et al.* (1988) was identified (underlined). The phosphorylatable Ser residue is indicated by an arrowhead. Note that the second half in this domain is more conservative than the first half between these two species. Two Met residues were found in *Mytilus* sequence, while only one was found in *Argopecten* (indicated by asterisks).

and the 3'-Adaptor Primer (Figure 2(a), clone 6). The rest of sequence was found to be identical with that of the MHC which (Figure 2(a), clone 5) was obtained by another 3'-RACE PCR experiment with an MHC-specific 5'-primer and the 3'-Adaptor Primer (see Figure 3). This result indicates that catchin has also a non-helical tailpiece containing the phosphorylatable Ser residue common to the MHC (Castellani *et al.*, 1988). In the coiled-coil rod region, the mussel and scallop sequences are 72% identical.

Catchin is produced by alternative RNA splicing of the MHC gene

A partial exon/intron map of the MHC gene of the scallop, *A. irradians* has previously been reported. This single muscle-specific MHC gene produces both the striated and catch type isoforms of MHC by alternative RNA splicing (Nyitray *et al.*, 1994). The boundary between the catchin-specific sequence and the sequence common to the MHC and catchin in *Mytilus* is located at the border of two exons on the scallop MHC gene map. We amplified and fully sequenced this part of the scallop MHC gene (Figure 6; GenBank accession no. AF183909). In the ~2.5 kb intron after exon 18 (nucleotide 3471 of the scallop MHC cDNA) an open reading frame was found which encodes similar amino acid sequence to the N-terminal unique domain of *Mytilus* catchin (Figure 6, upper map; see also Figure 5). Apparently, this open reading frame is spliced as an alternative exon to exon 19 of the MHC gene during processing of the primary message. It should be noted that two new exons were detected by sequencing the MHC gene (exons 17 and 18) which we failed to detect on the partial exon/intron map (Nyitray *et al.*, 1994). We also examined genomic DNA prepared from *M. galloprovincialis*. The corresponding intron was amplified by PCR using specific primers designed based on the cDNA sequence. A DNA fragment of ~4 kb was obtained and the exon of the N-terminal unique domain of catchin was identified within this intron of the *Mytilus* MHC gene (Figure 6, lower map). From these results, it is clear that catchin is produced from the MHC gene as an alternative transcriptional unit.

Discussion

Some molluscan muscles are catch muscles and others are not. To study differences between them can be a useful way to clarify the mechanism of the catch contraction. It is well known that a cAMP-dependent protein kinase is involved in relaxation of catch. Phosphorylation of paramyosin, MHC and RLC have previously been shown to occur *in vitro* (Cooley *et al.*, 1979; Castellani & Cohen, 1987; Sohma *et al.*, 1985). In the only *in vivo* study, Siegman *et al.* (1997, 1998) have found that the major phosphorylated protein during relaxation of *Mytilus* ABRM was a twitchin-related protein. However, all these proteins are common to both catch and non-catch muscles (Vibert *et al.*, 1993, 1996). In this report, we have identified a novel protein in molluscan muscles. Catchin, as we named it, is abundant only in catch muscles of all species examined so far. It is a heat and alcohol-resistant protein with rod-like shape as revealed by rotary shadowing (Yamada *et al.*, 1997). Catchin is

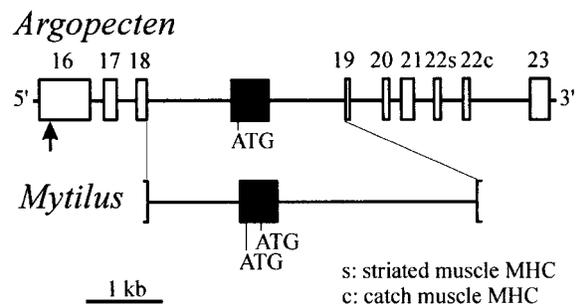


Figure 6. Exon/intron map showing the location of the alternative exon coding for the N-terminal unique domain of catchin. Part of the MHC gene (from exon 16 to exon 23) of *Argopecten* is shown in the upper map. Open boxes indicate exons coding for MHC sequences. Two additional small introns were identified compared to the previously published partial map (Nyitray *et al.*, 1994). The filled box is the exon coding the N-terminal unique domain of catchin, located in a large intron between exons 18 and 19. The arrow indicates the position of the head-rod junction. In *Mytilus*, the exon coding for the N-terminal domain of catchin was located in the corresponding intron of the MHC gene (lower map).

probably identical to a contaminating protein of ABRM myosin preparations, previously thought to be a degradation product of the MHC (Castellani & Cohen, 1987; Castellani *et al.*, 1988); to a heat-stable catch muscle component, tentatively identified as an invertebrate caldesmon-like protein (Bartegi *et al.*, 1989; Cszimadia *et al.*, 1994); and to an unknown heat-stable protein in molluscan catch muscles reported by others in preliminary form (Shelud'ko *et al.*, 1998; Kumeiko *et al.*, 1999). One can assume that catchin, as a catch muscle specific protein, plays some, perhaps a key role in the catch contraction of molluscan muscles.

The full-length cDNA of *M. galloprovincialis* catchin was cloned and sequenced. The encoded protein has three domains: the N-terminal domain, consisting of 156 amino acid residues, is unique to catchin and apparently does not form a coiled-coil structure; while the rest of the molecule is identical with the C-terminal two-thirds of the rod region of the MHC, consisting of an α -helical coiled-coil domain of 809 residues and a non-helical tailpiece domain of 21 residues. Because of the presence of the myosin rod sequence the catchin polypeptides must form dimeric coiled-coil protein as the MHCs do. Purified catchin forms filaments and it co-aggregates with rabbit skeletal muscle myosin filaments indicating that it is a component of the thick filament (Yamada *et al.*, 1997). Densitometry analysis shows that the estimated molar ratio of catchin to MHC in all catch muscle is as much as 1:1. Its precise location and distribution within the thick filament is being investigated.

The N-terminal domain may have some unique functions, since this domain is unique to catchin and does not show high similarity to any other known proteins. Close to the border of the unique N-terminal domain and the rod portion we have identified a peptide sequence which has previously been reported by Castellani *et al.* (1988) to contain a phosphorylatable Ser residue. In their report, this sequence was tentatively located in the non-helical tailpiece of MHC together with an additional phosphorylated peptide. Now it is clear that the first peptide comes from the N-terminal non-helical domain of catchin, and that the second peptide represents the C-terminal end of both catchin and the MHC. Thus, catchin has at least two phosphorylatable Ser residues, one in the N-terminal unique domain and the other in the C-terminal non-helical tailpiece domain common to the MHC. Phosphorylation of these Ser residues by an endogenous kinase must be some important but yet unknown functions.

It was noted earlier that in the *A. irradians* MHC gene some of the large "intervening" sequences may contain additional alternatively spliced exons (Nyitray *et al.*, 1994). Indeed, catchin is an alternative product of the MHC gene. The N-terminal unique domain is encoded within one of the large introns of the MHC gene (after exon 18) and spliced to the rod coding exon 19. The rod sequence common with the MHC starts near the

subfragment-2 hinge region within the rod (Ala1110 in the *A. irradians* MHC sequence). Interestingly, a myosin rod protein was also identified as an alternative product of the single *Drosophila* muscle MHC gene. This protein of 155 kDa in size was expressed abundantly in various muscles and in testis (Miedema, 1995; Standiford *et al.*, 1997). It has a unique 77 amino acid residue N-terminal domain that exactly replaces the MHC motor domain. Thus it has a full-length rod domain compared with the molluscan catchin in which the N-terminal domain is connected to the rod near the subfragment-2 hinge within the rod. There is no significant similarity between the amino acid sequences of the unique N-terminal domains except that both are rich in Pro, Ser and Thr residues. Another difference between the two internal transcripts is that the exon coding for the N-terminal domain in *Drosophila* rod protein is continuous in frame into the rod coding exon, while there is an additional intron in the molluscan genes. The presence of alternative transcriptional units in genes of thick filament proteins is not restricted to the MHC: *Drosophila* paramyosin gene encodes internally a truncated form, mini-paramyosin which also has a unique N-terminal non-helical domain (Maroto *et al.*, 1995, 1996).

What is the function of catchin and its unique N-terminal domain? One possibility is that the N-terminal domain contributes to the "setting" of the acto-myosin crossbridges during the catch state by either directly or by interacting with other thick filament associated proteins, particularly with twitchin. It was reported that twitchin was involved in regulation of catch contraction (Sieglman *et al.*, 1997, 1998). It would be interesting to see whether catchin is able to interact with twitchin by a phosphorylation dependent manner. If the N-terminal unique domain of catchin has an extended structure, it might tether actin to the thick filament in the catch state. Standiford *et al.* (1997) noted some relatedness between the N-terminal domain of the *Drosophila* rod protein and the N-terminal extensions of certain myosin light chains which might serve to tether the light chains to actin and modulate the contractile function. A similar function for the N-terminal domain of each myosin rod protein is conceivable. The role of phosphorylation of catchin might also be related to its putative role in the catch contraction or, alternatively it could be involved in the myofibrillogenesis of catch muscles. Catchin may also have a role in the formation of the peculiar structure of the large thick filaments of catch muscles compared to smaller thick filaments of more common non-catch muscles. Considering the organization of the thick filament it is worth to note that the coiled-coil portion of catchin has the same length (~125 nm) as that of paramyosin.

In summary, we have discovered a novel myosin rod-like protein, catchin, in molluscan catch muscles which is an alternative transcriptional product of the MHC gene. Apparently, catchin is a

major component of the thick filaments, and phosphorylatable by an endogenous kinase both in the unique N-terminal non-helical domain and in the C-terminal non-helical tailpiece domain. Now that the presence of catchin in catch muscles is evident, although its function is unclear yet, one has to take it into account as a "new player" for proposing models for the structure of catch muscle thick filaments and for the molecular basis of catch contraction.

Materials and Methods

Animals and muscle proteins

Blue mussels, *M. galloprovincialis*, and oysters, *Crassostrea* sp., were collected in the Inland Sea of Japan. Live scallops, *P. yessoensis* were purchased from a market near the Kansai Advanced Research Center. *A. irradians* and *P. magellanicus* muscles were gift from Dr A.G. Szent-Györgyi (Brandeis University, USA). Heat-stable protein fractions were prepared from these muscles by first homogenizing them in six volumes of 0.3 M KCl, 1 mM ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 0.5 mM MgCl₂, 50 mM imidazole (pH 7.0), then heated at 85 °C for four minutes. After cooling, the homogenate was centrifuged at 15,000 g for 30 minutes and the supernatant was dialyzed overnight against 1 mM EGTA, 1 mM dithiothreitol (DTT), 20 mM Tris-HCl (pH 7.5). The dialysate was centrifuged and the precipitate was analyzed by SDS-PAGE, together with total homogenates of the muscles. The gels were stained with Coomassie brilliant blue R-250, and densitometry was carried out with a Shimadzu CS-9300 densitometer.

Cloning experiments

Total RNA of *M. galloprovincialis* muscles was prepared essentially according to Chomczynski & Sacchi (1987). Complementary DNA was synthesized with a SuperScript II reverse transcriptase (Gibco BRL, Life Technologies, Inc., USA) using either an Oligo (dT) primer (for clones 1-3, 7 in Figure 2) or an Oligo (dT)-Adaptor Primer (TaKaRa Shuzo Co. Ltd., Japan; for clones 4-6, 8 in Figure 2). PCR experiments were performed with either a TaKaRa Ex Taq or a TaKaRa LA Taq DNA polymerase. The products were cloned into a pCR 2.1 cloning vector (Invitrogen) and sequenced with an automatic DNA sequencer (ABI PRISM 377 DNA Sequencer, Perkin Elmer). Clone 1 of *M. galloprovincialis* catch muscle MHC (Figure 2(a)) was amplified using a degenerate 5'-primer based on the partial amino acid sequence of the 120 kDa and 112 kDa proteins (Yamada *et al.*, 1997) and a specific 3'-primer designed based on a partial cDNA sequence of *M. edulis* MHC (L.N., unpublished results; GenBank accession no. U40036). Clone 2 was amplified with a degenerate 5'-primer designed according to a highly conservative amino acid sequence in the myosin head and a specific 3'-primer based on the sequence of clone 1. Clone 3 was constructed the same way. We carried out 3'-RACE PCR experiments to make clones 4-6 using specific 5'-primers and a 3'-Adaptor Primer. An MHC cDNA fragment from PRM was also cloned (Figure 2(a), clone 7) using the same primers as used for clone 3. The 3'-end of the PRM MHC cDNA (clone 8) was amplified by a 3'-RACE PCR experiment

with a specific 5'-primer designed based on the sequence of clone 7 and the 3'-Adaptor Primer.

In order to get the 5'-end of the catchin cDNA, we performed a 5'-RACE PCR experiment using a TaKaRa 5'-Full RACE Core Set and a SuperScript II reverse transcriptase (Figure 2(d)), essentially according to the manufacturer's instructions. The product was cloned and sequenced as described above.

A genomic fragment of the scallop (*Argopecten*) MHC gene was obtained by PCR amplification using cDNA specific primers annealing to the sequences 3200-3222 and 3577-3557 (Nitray *et al.*, 1991, 1994; GenBank accession no. X55714).

Northern blotting

Digoxigenine (DIG)-labeled RNA probes (Figure 2(c)) for Northern blot analysis were synthesized with a DIG RNA Labeling Kit (SP6/T7) (Boehringer Mannheim). Total RNAs from *Mytilus* muscles were electrophoresed and transferred to a Hybond-N+ nylon membrane (Amersham Life Science Ltd., UK) essentially as described by Sambrook *et al.* (1989). Hybridization of the labeled probes and conjugation with an anti-DIG antibody-peroxidase complex were carried out with a DIG Nucleic Acid Detection Kit (Boehringer Mannheim) according to the instruction manual. The peroxidase complex on the nylon membrane was visualized with ELC Western blotting detection reagents (Amersham).

Nucleotide sequence accession numbers

The nucleotide sequences reported here have been submitted to the GenBank Nucleotide Sequence Database under accession numbers AJ249991 (*M. galloprovincialis* catch muscle MHC cDNA), AJ249992 (*M. galloprovincialis* PRM MHC cDNA), AJ249993 (*M. galloprovincialis* catchin cDNA), and AF183909 (*A. irradians* MHC/catchin genomic DNA).

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References

- Achazi, R. K. (1979). Phosphorylation of molluscan paromyosin. *Pflügers Arch.* **379**, 197-201.
- Atsumi, S. & Sugi, H. (1976). Localization of calcium-accumulating structures in the anterior byssal retractor muscle of *Mytilus edulis* and their role in the regulation of active and catch contractions. *J. Physiol.* **257**, 549-560.
- Bartegi, A., Fattoum, A., Dagorn, C., Gabrion, J. & Kassab, R. (1989). Isolation, characterization, and

- immunocytochemical localization of caldesmon-like protein from molluscan striated muscle. *Eur. J. Biochem.* **185**, 589-595.
- Butler, T. M., Moores, S. U., Li, C., Narayan, S. & Siegman, M. J. (1998). Regulation of catch muscle by twitchin phosphorylation: effects on force, ATPase, and shortening. *Biophys. J.* **75**, 1904-1914.
- Castellani, L. & Cohen, C. (1987). Myosin rod phosphorylation and the catch state of molluscan muscles. *Science*, **235**, 334-337.
- Castellani, L. & Cohen, C. (1992). A calcineurin-like phosphatase is required for catch contraction. *FEBS Letters*, **309**, 321-326.
- Castellani, L., Elliot, B. W., Jr & Cohen, C. (1988). Phosphorylatable serine residues are located in a non-helical tailpiece of a catch muscle myosin. *J. Muscle Res. Cell Motil.* **9**, 533-540.
- Chantler, P. D. (1991). The structure and function of scallop adductor muscles. In *Scallops: Biology, Ecology and Aquaculture* (Shumway, S. E., ed.), pp. 225-305, Elsevier, Amsterdam.
- Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.
- Cohen, C. (1982). Matching molecules in the catch mechanism. *Proc. Natl Acad. Sci. USA*, **79**, 3176-3178.
- Cole, R. A. & Twarog, B. M. (1972). Relaxation of catch in a molluscan smooth muscle. I. Effects of drugs which act on the adenyl cyclase system. *Comp. Biochem. Physiol. sect. A*, **43**, 321-330.
- Cooley, L. B., Johnson, W. H. & Krause, S. (1979). Phosphorylation of paramyosin and its possible role in the catch mechanism. *J. Biol. Chem.* **254**, 2195-2198.
- Csizmadia, A. M., Bonet-Kerrache, A., Nyitray, L. & Mornet, D. (1994). Purification and properties of caldesmon-like protein from molluscan smooth muscle. *Comp. Biochem. Physiol. sect. B*, **108**, 59-63.
- Ishii, N. & Takahashi, K. (1981). Mechanical properties and fine structure of the pedal retractor muscle of *Mytilus edulis*. *Comp. Biochem. Physiol. sect. A*, **70**, 275-283.
- Ishii, N., Simpson, A. W. M. & Ashley, C. C. (1989). Free calcium at rest during "catch" in single smooth muscle cells. *Science*, **243**, 1367-1368.
- Kendrick-Jones, J., Lehman, W. & Szent-Györgyi, A. G. (1970). Regulation in molluscan muscles. *J. Mol. Biol.* **54**, 313-326.
- Kondo, S. & Morita, F. (1981). Smooth muscle of scallop adductor contains at least two kinds of myosin. *J. Biochem.* **90**, 673-681.
- Kumeiko, V. V., Plotnikov, S. V., Moroz, E. S. & Korchagin, V. P. (1999). A new heat stable regulatory protein of scallop catch muscle that interacts with thick filaments. *J. Muscle Res. Cell Motil.* **20**, 73.
- Kurzawa-Goertz, S. E., Perreault-Micale, C. L., Trybus, K. M., Szent-Györgyi, A. G. & Geeves, M. A. (1998). Loop I can modulate ADP affinity, ATPase activity, and motility of different scallop myosins. Transient kinetic analysis of S1 isoforms. *Biochemistry*, **37**, 7517-7525.
- Lupas, A., Van Dyke, M. & Stock, J. (1991). Predicting coiled coils from protein sequences. *Science*, **252**, 1162-1164.
- Maroto, M., Arredondo, J. J., San Roman, M., Marco, R. & Cervera, M. (1995). Analysis of the paramyosin/miniparamyosin gene. Miniparamyosin is an independently transcribed, distinct paramyosin isoform, widely distributed in invertebrates. *J. Biol. Chem.* **270**, 4375-4382.
- Maroto, M., Arredondo, J. J., Goulding, D., Marco, R., Bullard, B. & Cervera, M. (1996). *Drosophila* paramyosin/miniparamyosin gene products show a large diversity in quantity, localization, and isoform pattern: a possible role in muscle maturation and function. *J. Cell Biol.* **134**, 81-92.
- Miedema, K., Hanske, M., Akhmanova, A., Bindels, P. & Hennig, W. (1995). Minor-myosin, a novel myosin isoform synthesized preferentially in *Drosophila* testis is encoded by the muscle myosin heavy chain gene. *Mech. Dev.* **51**, 67-81.
- Nyitray, L., Goodwin, E. B. & Szent-Györgyi, A. G. (1991). Complete primary structure of a scallop striated muscle myosin heavy chain. *J. Biol. Chem.* **266**, 18469-18476.
- Nyitray, L., Jancsó, Á., Ochiai, Y., Gráf, L. & Szent-Györgyi, A. G. (1994). Scallop striated and smooth muscle myosin heavy-chain isoforms are produced by alternative RNA splicing from a single gene. *Proc. Natl Acad. Sci. USA*, **91**, 12686-12690.
- Perreault-Micale, C. L., Jancsó, Á. & Szent-Györgyi, A. G. (1996a). Isoforms of the essential and regulatory light chains of *Placopecten* striated and catch muscle myosins. *J. Muscle Res. Cell Motil.* **17**, 533-542.
- Perreault-Micale, C. L., Kalabokins, V. N., Nyitray, L. & Szent-Györgyi, A. G. (1996b). Sequence variations in the surface loop near the nucleotide binding site modulate the ATP turnover rates of molluscan myosins. *J. Muscle Res. Cell Motil.* **17**, 543-553.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning*, 2nd edit., Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
- Shelud'ko, N. S., Tuturova, K. P., Permyakova, T. V. & Orlova, A. A. (1998). Thick filaments in smooth muscles of bivalve molluscs contain an unknown protein. *Biophys. J.* **74**, A262.
- Siegman, M. J., Mooers, S. U., Li, C., Narayan, S., Trinkle-Mulcahy, L., Watabe, S., Hartshorne, D. J. & Butler, T. M. (1997). Phosphorylation of a high molecular weight (~600 kDa) protein regulates catch in invertebrate smooth muscle. *J. Muscle Res. Cell Motil.* **18**, 655-670.
- Siegman, M. J., Funabara, D., Kinoshita, S., Watabe, S., Hartshorne, D. J. & Butler, T. M. (1998). Phosphorylation of a twitchin-related protein controls catch and calcium sensitivity of force production in invertebrate smooth muscle. *Proc. Natl Acad. Sci. USA*, **95**, 5383-5388.
- Sohma, H., Yazawa, M. & Morita, F. (1985). Phosphorylation of regulatory light chain a (RLC-a) in smooth muscle myosin of scallop, *Patinopecten yessoensis*. *J. Biochem.* **98**, 569-572.
- Standiford, D. M., Davis, M. B., Miedema, K., Franzini-Armstrong, C. & Emerson, C. P., Jr (1997). Myosin rod protein: a novel thick filament component of *Drosophila* muscle. *J. Mol. Biol.* **265**, 40-55.
- Szent-Györgyi, A. G., Cohen, C. & Kendrick-Jones, J. (1971). Paramyosin and the filaments of molluscan "catch" muscles. II. Native filaments: isolation and characterization. *J. Mol. Biol.* **56**, 239-258.
- Twarog, B. M. (1979). The nature of catch and its control. In *Motility in Cell Function* (Pepe, F. A., Sanger, J. W. & Nachmias, V. T., eds), pp. 231-241, Academic Press, New York.
- Vibert, P., Edelstein, S. M., Castellani, L. & Elliot, B. W., Jr (1993). Mini-titins in striated and smooth mollus-

- can muscles: structure, location, and immunological crossreactivity. *J. Muscle Res. Cell Motil.* **14**, 598-670.
- Vibert, P., York, M. L., Castellani, L., Edelstein, S., Elliot, B. & Nyitray, L. (1996). Structure and distribution of mini-titins. *Advan. Biophys.* **33**, 199-209.
- Yamada, A., Ishii, N., Shimmen, T. & Takahashi, K. (1989). Mg-ATPase activity and motility of native thick filaments isolated from the anterior byssus retractor muscle of *Mytilus edulis*. *J. Muscle Res. Cell Motil.* **10**, 124-134.
- Yamada, A., Yoshio, M. & Nakayama, H. (1997). Bi-directional movement of actin filaments along long bipolar tracks of oriented rabbit skeletal muscle myosin molecules. *FEBS Letters*, **409**, 380-384.
- Yamada, A., Yoshio, M. & Nyitray, L. (1999). Catchin, a novel contractile protein in molluscan catch muscles, is produced from an alternative transcriptional unit of the myosin heavy chain gene. *J. Muscle Res. Cell Motil.* **20**, 108.

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