Immunolocalization of calcium vector protein and its target protein in amphioxus

Valette, Laurence Agnes; Comte, Michelle; Chaponnier, Christine; Cox, Jos Adrian

Histochemistry (1993) 100:73–81

Histochemistry

Immunolocalization of calcium vector protein and its target protein in amphioxus

Laurence Valette-Talbi[1], **Michelle Comte**[1], **Christine Chaponnier**[2], **Jos A. Cox**[1]

[1] Department of Biochemistry 30, Quai Ernest-Ansermet, University of Geneva, CH-1211 Geneva 4, Switzerland
[2] Department of Pathology, University of Geneva, CH-1211 Geneva 4, Switzerland

Accepted: 21 April 1993

Abstract. Three proteins, sarcoplasmic Ca^{2+}-binding protein (SCP), Ca^{2+} vector protein (CaVP) and its target protein (CaVPT), are found abundantly in the higher invertebrate amphioxus. Whereas the function of SCP is likely to be related to Ca^{2+} and Mg^{2+} buffering, that of the latter two proteins, apparently linked together, is still not clear. In this study, affinity-purified polyclonal antibodies to these three proteins were used to study the extractability under physiological ionic conditions, the distribution in different tissues and the immunocytochemical localization in striated muscle. Our data show that SCP is essentially cytosolic whereas CaVP and CaVPT are partially associated with non-soluble components in amphioxus tissues. The tissue distribution, studied in transverse sections, shows that SCP is merely confined to striated muscle, whereas CaVP and CaVPT are also abundant in other tissues such as the spinal chord and the gonads. Thus the protein pair CaVP/CaVPT is likely to serve a general role in many tissues; however, no strict correlation was found in the distribution of the latter two proteins, suggesting that they may function independently. The detailed cytochemical localization of the three proteins in longitudinal sections of striated muscle revealed a discrete striation pattern in addition to a diffuse background. For SCP these striations are coincident with the Z line. The immunostaining for CaVP shows intense striations at the level of the Z lines alternating with weak striations at the M lines. For CaVPT the striations at the Z and M line are more or less of equal intensity, leading to a pattern with a 1μm periodicity. The data lead to the conclusion that CaVP and CaVPT can form dynamic complexes with structural components of the sarcomere.

Introduction

The muscle of amphioxus, commonly called the lancelet, is very rich in acidic Ca^{2+}-binding proteins belonging to the EF-hand family. One type, named sarcoplasmic Ca^{2+}-binding protein (SCP), is abundant and easily extractable in physiological ionic conditions (Kohler et al. 1979). Homologous members of this family have been found in other invertebrates such as crustaceans, annelids and molluscs (for review see Cox 1990). Since these proteins do not interact with target proteins or with hydrophobic matrices, we assumed that their main role is the buffering of free Ca^{2+} and Mg^{2+} in muscle (Wnuk et al. 1982). SCP shows pronounced polymorphism due to a limited number of point mutations in a segment of 17 residues (Takagi et al. 1986, 1992; Takagi and Cox 1990a).

A second type of Ca^{2+}-binding protein, also abundant in amphioxus muscle, is the so-called Ca^{2+} vector protein (CaVP). Although CaVP belongs to the so-called EF-hand family, its amino acid sequence (Kobayashi et al. 1987) is unique and no homologous Ca^{2+}-binding protein has yet been described in any other invertebrate or vertebrate phylum (Moncrief et al. 1990; Nakayama et al. 1992). CaVP clearly shows the characteristics of a Ca^{2+}-dependent activator (Cox et al. 1990) and is associated in vivo to a target protein of 26 kDa, designated CaVPT (target protein of CaVP). The complex CaVP-CaVPT is quite easily extracted at physiological ionic conditions. It can be dissociated by 6 *M* urea, heating to 70° C or electrical current in the presence of EGTA (Cox 1986), but not by EGTA alone (Cox JA, unpublished results). Some clues concerning the function of the CaVP-CaVPT complex can be inferred from the amino acid sequence of CaVPT (Takagi and Cox 1990b). In addition to a CaVP-binding domain, which resembles a particular type of calmodulin-binding domain in unconventional myosins (Espreafico et al. 1992), CaVPT possesses two copies of a sequence motif of 100 residues. This motif, called the IgII domain, is also present in some proteins interacting with myosin or with each other, namely titin (Labeit et al. 1990), twitchin (Benian et al. 1989), C protein (Einheber and Fischman 1990), myosin light chain kinase (Olson et al. 1990) and telokin (Ito et al. 1989). The presence of this motif in CaVPT suggests that the protein recognizes the myosin rod or

Correspondence to: J.A. Cox

other proteins with IgII domains. To investigate the function of the CaVP-CaVPT complex, we report here on the occurrence of CaVP and CaVPT in different organs of amphioxus, on the extractability of these proteins and on the intracellular localization in muscle. For the sake of comparison, parallel experiments were carried out on SCP, believed to be a soluble muscular protein.

Materials and methods

Purification of SCP, CaVP and CaVPT from amphioxus

Proteins were extracted from frozen amphioxus (*Branchiostoma lanceolatum*; obtained from Laboratoire Arago, Banyuls-sur-Mer, France) as previously described (Cox 1986). After DEAE-52 cellulose and Sephacryl S-200 chromatography, fractions containing the complex CaVP-CaVPT and free CaVP were localized by 12.5% polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS; Laemmli 1970). The purified complex was heated at 75° C for 2 min and the suspension clarified by centrifugation at 10,000 rpm for 20 min. The supernatant contains the thermostable CaVP, which was further purified by Sephadex G-75 chromatography; the pellet consisting mostly of CaVPT was dissolved in 6 *M* urea and chromatographed on a calmodulin-agarose column (prepared according to the mini-leak technique; Biocarb, Lund, Sweden) as described by Takagi and Cox (1990).

The SCP containing fractions of the first DEAE-52 cellulose chromatographic separation were further purified as previously described (Takagi et al. 1986). In this work we have used only the antibody against type I SCP. The other types of SCP react equally well with this polyclonal antibody (Cox JA, unpublished results). Bovine brain calmodulin was purified as described by Gopalakrishna and Anderson (1982). Amphioxus troponin C was purified using a new method established in our laboratory (Takagi T, Petrova, Comte M, Kuster T, Heizmann CW, Cox JA, manuscript in preparation).

Production and immunopurification of antisera

Antisera used in this study were polyclonal rabbit anti-SCP, rabbit anti-CaVPT (the antigen which eluted with 0.6 M KCl from the calmodulin-agarose column), chicken anti-CaVP and monoclonal mouse IgM anti rabbit α-actinin (Bio-Makor, Rehovot, Israel). After the standard purification procedure described above, CaVP and CaVPT were further purified by electrophoresis in SDS. The Coomassie-stained bands were excised and homogenized in Freund's adjuvant. Immunization was performed by multiple intradermal injections in New Zealand white rabbits or chicken of 250 µg pure antigen, according to standard procedures (Harlow and Lane 1988). Purification of the antibodies from the immunoreactive sera was done by affinity chromatography on the pure antigen coupled to activated CNBr Sepharose 4B (1–2.5 mg protein per ml of resin). The specific antibodies were eluted with 0.2 *M* glycine-HCl, pH 2.8, neutralized, dialysed against phosphate-buffered saline, concentrated and kept in 3 m*M* NaN_3. The protein concentration was determined spectrophotometrically using an extinction coefficient, $\varepsilon_{1\%}$, of 13.5 at 280 nm.

Electrophoresis, Western blotting and antigen detection

Pure antigens and/or amphioxus protein extracts, as well as the low range pre-stained protein standards (Bio-Rad, Richmond, Calif., USA), were submitted to 12.5% polyacrylamide electrophoresis in the presence of SDS according to Laemmli (1970). Proteins were transferred onto nitrocellulose paper and the efficiency of transfer was ascertained with the pre-stained standards. The electrophoretic blots were soaked in 10% milk in phosphate buffer for 30 min to saturate free protein-binding sites, then incubated for 90 min with 2–4 µg/ml antibodies in 10% milk, 3% Tween in phosphate buffer. The blots were washed three times with 3% Tween in phosphate buffer and incubated for 90 min at room temperature with the same solution containing 1 µCi/10 ml [^{125}I]protein A (Amersham, Buckinghamshire, UK). Blots were washed five times with 3% Tween in phosphate buffer, dried and autoradiographed. Alternatively, the nitrocellulose blots were incubated for 60 min with 15–30 ng/ml of the specific antibodies, washed twice with 0.2% caseine + 0.1% Tween in phosphate buffer and then incubated for 30 min with 0.1 µg/ml anti-rabbit IgG conjugated to alkaline phosphatase. The activity of the latter enzyme was detected by means of the chemiluminescent substrate adamantyl-1,2-dioxethane-phosphate (Tropix, Bedford, Mass., USA), according to a method adapted from the protocol described by Gillespie and Hudspeth (1991). In the case of the antibody raised in chicken, an intermediate rabbit anti-chicken IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) was used since protein A does not stain the avian immunoglobulins (Surolia et al. 1982).

Immunofluorescence staining

Immunofluorescence staining with the different antibodies was performed on transverse sections of the entire animal and on longitudinal sections of muscle. Cryostat sections 3 µm thick were fixed in acetone or in 1% paraformaldehyde and dried for 2 h at room temperature. Sections were then incubated at room temperature with the first antibody for 30 min. After rinsing three times in phosphate buffer, the appropriate second antibody was applied and the sections incubated as before. The following second antibodies, all from Nordic Immunological Laboratories (Tilburg, The Netherlands), were used in this study: fluorescein isothiocyanate (FITC)-labelled goat anti-chick IgG, FITC-labelled goat anti-mouse IgG, tetramethylrhodamine isothiocyanate (TRITC)-labelled goat anti-rabbit IgG and TRITC-labelled goat anti-mouse IgG. For double-labelled immunofluorescence microscopy, both primary and secondary antibodies were added simultaneously. After rinsing in phosphate buffer as before, the sections were mounted in buffered polyvinylalcohol (Lennette 1978) and observed with a Zeiss Axiophot equipped with epi-illumination and specific filters for rhodamine and fluorescein (Carl Zeiss, Oberkochen, Germany). Controls were performed using non-immune IgG in place of the primary antibody or the fractions of the sera not retained on the immobilized antigens. Photographs were taken on T-Max Kodak black and white film (Kodak, UK).

Internal anatomy of amphioxus

Although amphioxus is genetically an invertebrate, its anatomy shows much resemblance with that of the fishes (Fig. 1). The lateral body wall muscle consists of 64 segmental V-shaped myotomes, separated by the myosepts. The notochord bears some morphological and functional resemblance to the chord in lower vertebrates: it is constituted of a stack of flattened lamellae and surrounded by a thick collagenous sheath. These lamellae are cross-striated muscle cells with a sarcomere length of 33 µm and able to contract upon electric stimulation. Contraction of these muscle cells reduces the transverse diameter and stiffens the notochord (Flood 1975). The spinal chord is composed of longitudinally running fibres with profiles of between 0.2 and 0.4 µm (Guthrie 1975). In the coelomic sac are situated four organs displaying some homology to those of vertebrates: the digestive caecum which is considered to be the precursor of liver and pancreas, the endostyle which corresponds to the thyroid gland, the gonads and the pharynx. The pharynx is septated by the pharyngeal bars, which retain the food but let the water enter the peribranchial cavity (Welsch 1975). The dorsal roof of the pharynx is formed by the epibranchial groove, rich in various lytic enzymes. The endostyle contains six different cell

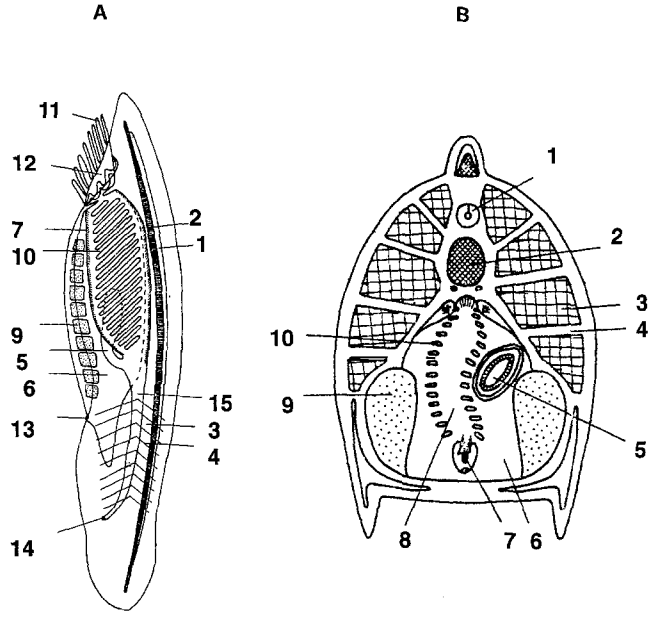

Fig. 1A, B. Internal anatomy of amphioxus. **A** Longitudinal section; **B** transverse section at the level of the pharynx. *1*, Spinal chord; *2*, notochord; *3*, myomeric muscle; *4*, myosept; *5*, digestive caecum; *6*, peribranchial cavity; *7*, endostyle; *8*, pharynx; *9*, gonad; *10*, pharnygeal bar; *11*, buccal cirrus; *12*, buccal cavity; *13*, abdominal pore; *14*, anus; *15*, intestine

zones; the lateral zones are involved in iodine binding (Thorpe and Thorndyke 1975) and therefore supposed to contain secretory cells.

Results

Immunochemical characterization of anti-SCP, anti-CaVP and anti-CaVPT antibodies

Immunoblotting after electrophoresis in SDS of the total protein content of amphioxus (from an animal directly homogenized in 2% SDS and clarified by centrifugation) and of the pure antigens showed that they reacted with a single band (Fig. 2). This indicates that they are highly specific and suited for the further investigations. Since calmodulin and troponin C display pronounced sequence similarity with CaVP and SCP and have similar molecular weights, the antibodies against the latter two proteins was also tested against both bovine calmodulin, which is likely to be very similar to amphioxus calmodulin (Wylie and Vanaman 1988), and amphioxus troponin C. Figure 2 shows that the two Ca^{2+}-binding proteins SCP and CaVP do also not noticeably cross-react with calmodulin and troponin C. Occasionally anti-CaVPT reacted with diffuse bands of low molecular weight, suggesting that proteolysis occurs.

Extractability of the amphioxus proteins SCP, CaVP and CaVPT

To determine to what extent CaVP and CaVPT are soluble in the tissues, 7.5 g of whole animals were extracted 12 times, each with 15 ml of 20 m*M* *Tris*-HCl, pH 7.5, 100 m*M* KCl, 7.5 m*M* mercaptoethanol, 10 μ*M* $CaCl_2$, 0.1 mg/l pepstatin A, 1 mg/ml leupeptin and 70 mg/l phenylmethanesulphonyl fluoride and containing either 10 μ*M* $CaCl_2$ or 1 m*M* EGTA. Immunoblotting of the extracts showed that SCP, CaVP and CaVPT had different patterns of extraction (Fig. 3). Nearly all SCP was extracted after the fourth step as expected for a soluble protein. CaVP and CaVPT also were merely found in the supernatants of the first extractions, but significant amounts remained and were extracted slowly and concomitantly in supernatants 5–8. The final pellet after 12 extractions did not contain any SCP or CaVP, but still contained significant amounts of CaVPT. When the extractions were carried out with buffer containing EGTA instead of Ca^{2+}, the extraction profile was very similar to that of Fig. 3, including the small amount of pellet-associated CaVPT (data not shown).

A more quantitative determination of the different antigens in the first six extracts with enzyme-linked immunosorbent assay showed that the concentrations of SCP, CaVP and CaVPT in the successive extractions decreased according to the partition equation:

$$Q_n = K \cdot Q_t (1+K)^n,$$

where Q_n is the concentration of antigen obtained at the nth extraction, Q_t the total extractable antigen and K the partition coefficient. The values of K are 4.7, 2.7 and 2.0 for SCP, CaVPT and CaVP, respectively. For the sake of comparison, the partition coefficient for troponin C extraction from rabbit skeletal muscle myofibrils amounts to 1.4 (Cox et al. 1981). Taking account of the experimental conditions and the volume of the pellet, the value of 4.7 for SCP corresponds to that of a completely soluble protein, which is in agreement with previous studies on this class of proteins (Cox 1990). Our data suggest that CaVP and CaVPT display some affinity for insoluble components of amphioxus muscle. The total extractable amounts of SCP, CaVP and CaVPT are 12, 22 and 15 mg/g of muscle. The amount of CaVPT remaining in the pellet is approximately 3% of the total amount.

Tissue distribution of SCP, CaVP and CaVPT by immunohistochemical localization

Figure 4 shows the transverse section of amphioxus at the level of the digestive caecum, stained with antibodies against SCP, CaVP and CaVPT. Control experiments with non-immune IgG and the protein fraction of antisera not retained on the immobilized antigens revealed weak staining with the epidermal cells, with the collagenous notochordal sheath (skeletogenic layer) and with the primary pharyngeal bars, whose coelomic channels contain a pigment (data not shown). The specific immune reaction with anti-SCP is most restricted to the lateral and pterygial muscle and to the notochord. Anti-SCP reactivity is absent from the spinal chord, the gonads, the endostyle and the digestive caecum. The anti-CaVP immunoreactivity shows a more general distribu-

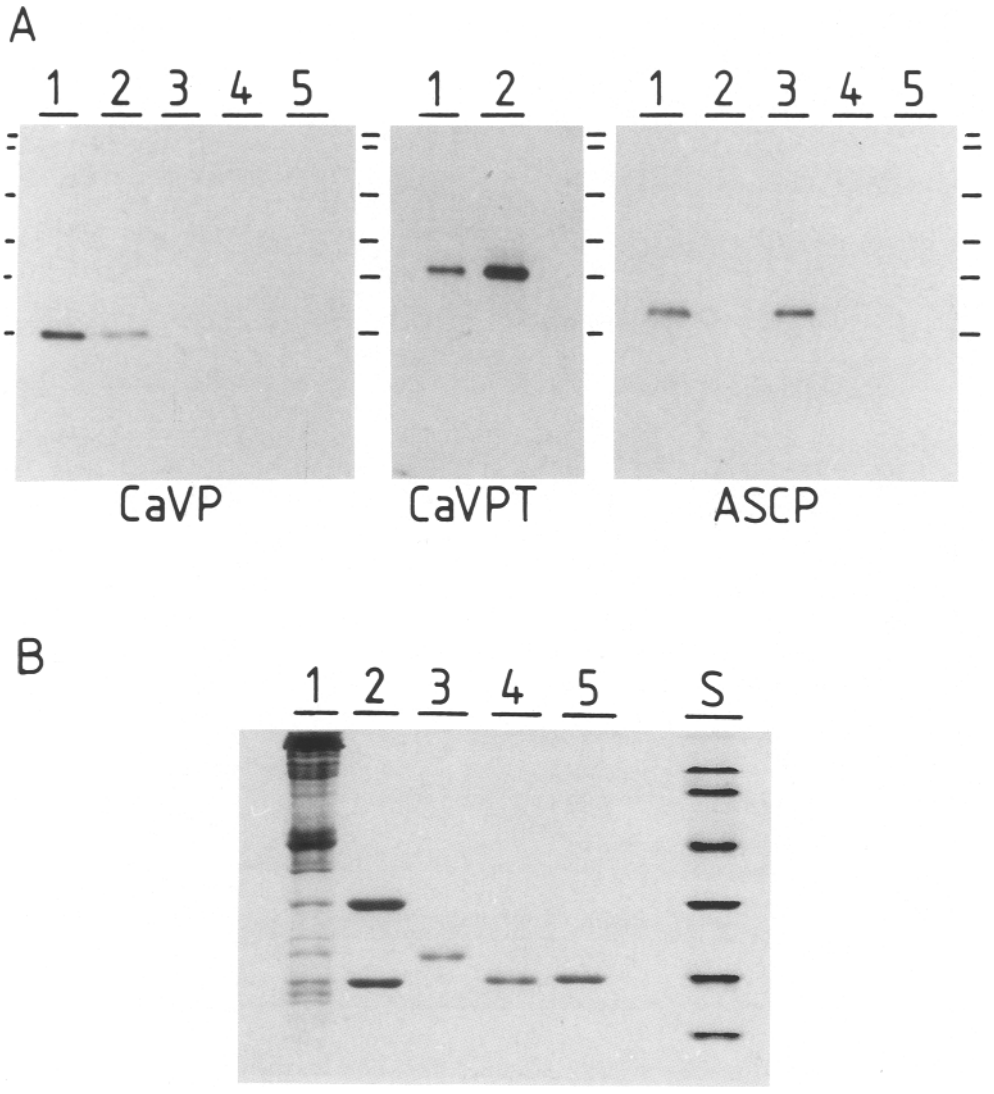

Fig. 2A, B. Specificity of the antibodies against calcium vector protein (CaVP), target protein of CaVP (CaVPT) and amphioxus sarcoplasmic calcium-binding protein (SCP; labelled ASCP to indicate the origin). **A** Immunoblots of: *lane 1*, 50 nl (0.25 g tissue/ml) amphioxus homogenate; *lane 2*, 50 ng of the CaVPT-CaVP complex; *lane 3*, 20 ng SCP; *lane 4*, 20 ng amphioxus troponin C; and *lane 5*, 20 ng bovine brain calmodulin. The chemiluminescence detection method was used (see the Materials and methods). *Horizontal marks* indicate the mobilities of the prestained molecular weight standards (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa). **B** Coomassie blue-stained gels containing: *lane 1*, 1.5 µl homogenate; *lane 2*, 5 µg CaVPT-CaVP complex; *lane 3*, 2 µg amphioxus SCP; *lane 4*, 2 µg amphioxus troponin C; and *lane 5*, 2 µg calmodulin. *Lane S* contained molecular weight standards (Bio-Rad) of 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa, respectively

tion than that of SCP. It is not only found in the different types of muscle (lateral, pterygial and notochordal), but also in the epiderm, gonads and the spinal chord. In the gonads the staining shows a flamboyant pattern, whereas in the spinal chord it looks like a fine network of fibres. The digestive caecum is also slightly immunostained. Anti-CaVPT shows a staining pattern resembling that of CaVP, although the CaVPT staining intensities in the notochord, gonads and spinal chord are much lower than those in skeletal muscle. The lateral ciliated cells of the endostyle (arrow in Fig. 4c), a population of cells rich in secretory granules, are more intensely stained with anti-CaVPT than with anti-CaVP.

Immunocytochemical localization in muscle

Detailed immunocytochemical localization with the antibodies in muscle revealed that in longitudinal sections SCP, CaVP and CaVPT show, in addition to a generally diffuse background, a discrete striation pattern (Fig. 5). Control experiments with non-immune IgG or serum fractions not retained on immobilized antigens yielded for all proteins uniform, nearly black pictures, except for weakly fluorescent borders outside the muscle tissues (not shown). The correlation of the striations with the sarcomere structure was facilitated by the fact that the antibody against rabbit α-actinin stained the Z lines in amphioxus muscle longitudinal sections (Fig. 5a, c, e). For SCP the striations are coincident with those of α-actinin, i.e. with a periodicity of 2 µm (Fig. 5b). Staining with anti-CaVP revealed a complex striation pattern (Fig. 5d) with two types of lines: narrow, intense lines corresponding to those of α-actinin and less intense immunoreactive lines in the middle of the sarcomeres. For CaVPT the longitudinal sections show a pattern of equal-intensity lines with a periodicity of 1 µm. For both CaVP and CaVPT the intensity of the M-band staining is somewhat variable depending upon the preparation. These data suggest that, in addition to the soluble CaVP-CaVPT complex, which yields a high fluorescence background, the proteins are concentrated at the Z line and in the M band.

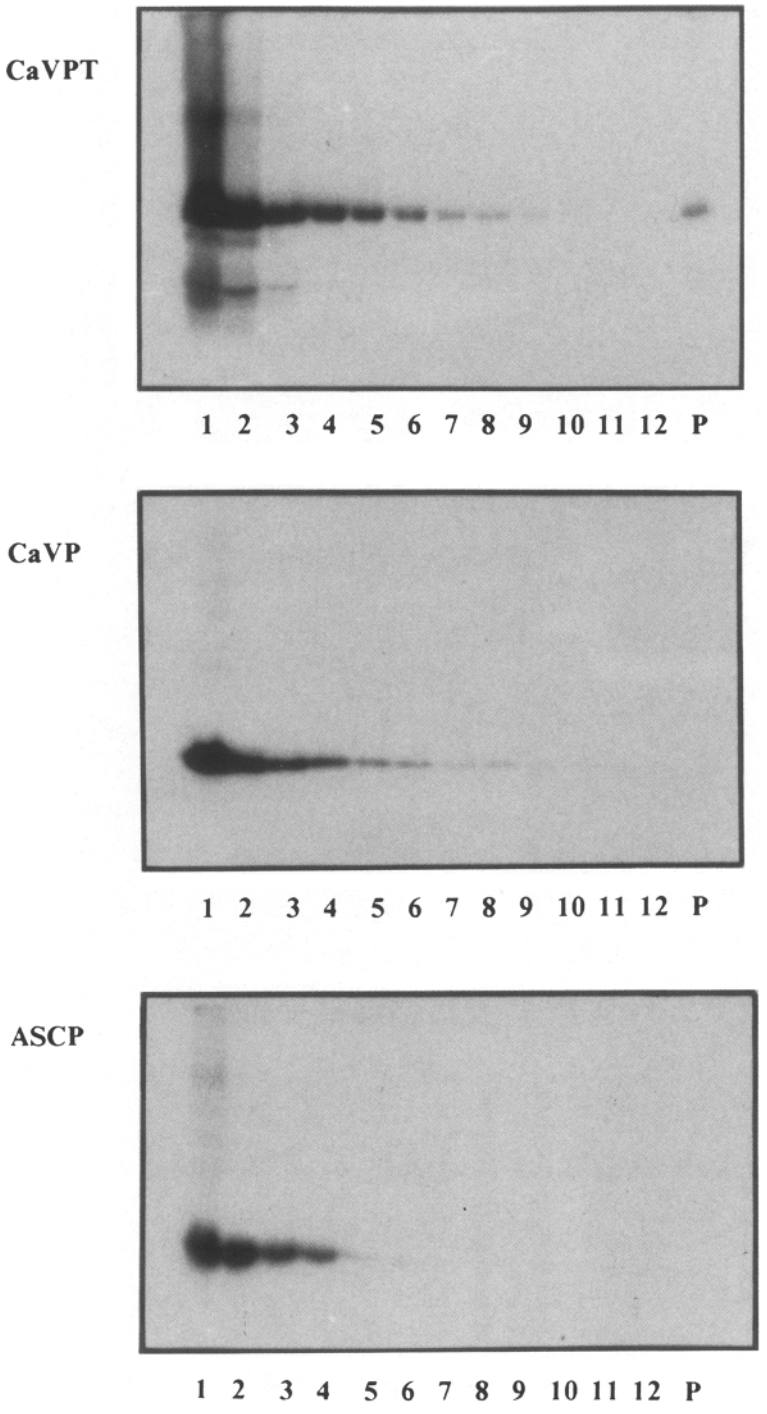

Fig. 3. Immunoblots after electrophoresis in sodium dodecyl sulphate (SDS) of twelve successive extracts (numbered 1–12) of amphioxus proteins. A 2.5 aliquot of the supernatants 1 to 12 and the SDS-resuspended pellets (*P*) were applied and the blots were incubated with anti-SCP antibody (labelled ASCP), anti-CaVP antibody or anti-CaVPT antibody. The [^{125}I]protein A detection method was used

Discussion

From the immunolocalization studies reported here it appears that CaVP and CaVPT show a wide tissue distribution, whereas SCP is mostly confined to the muscular organs. The latter is present in both the classical type (myomeric and pterygial muscle) and the unusual type (notochord). Qualitatively, but not quantitatively, the tissue distribution of CaVP and CaVPT is similar, suggesting that both constitute one heterodimeric protein. However there are three lines of evidence against the hypothesis of CaVP and CaVPT as subunits of one protein. Firstly, the intensities of staining of the respective antibodies in different organs, compared to those in muscle, indicate that the content of CaVP is much higher in gonads, notochord and spinal chord whereas that of CaVPT is much higher in some cells of the endostyle. Secondly, the extractabilities of CaVP and CaVPT are

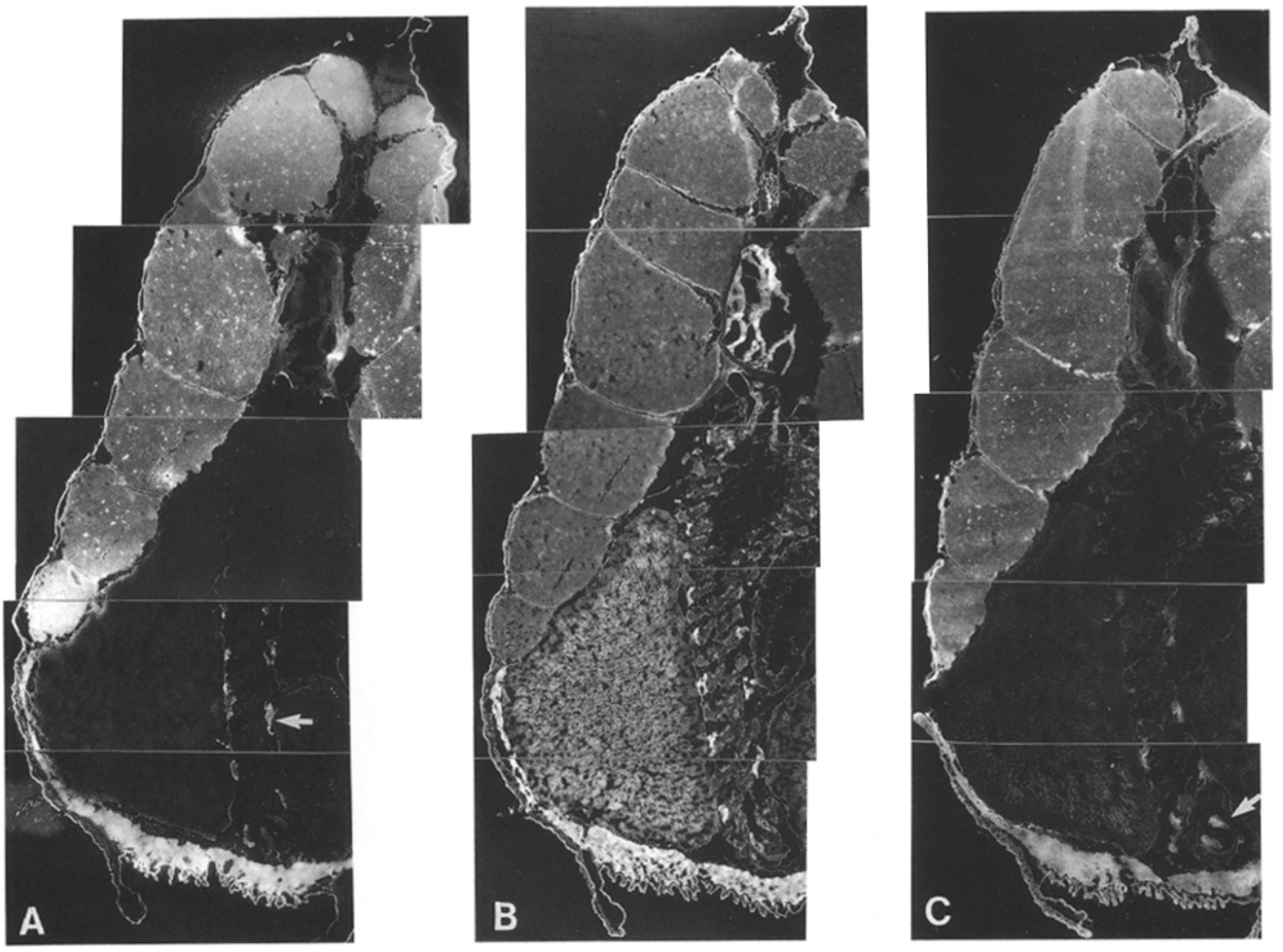

Fig. 4A–C. Photographs showing immunofluorescence of transverse sections of the lancelet at the level of the posterior border of the pharynx. **A** Anti-SCP; **B** anti-CaVP; **C** anti-CaVPT. 1 cm = 50 μm. It must be noted that the intensities of the pharyngeal bars (*arrow* in **A**) are due to autofluorescence. The lateral ciliated cells of the endostyle, stained specifically by anti-CaVPT, are indicated by a *arrow* in **C**

different, indicating that the proteins are associated with different insoluble structures, and thirdly, the contents of CaVP and CaVPT amount to 1.2 and 0.6 millimoles per kilogram of animals, i.e. there is an excess of CaVP. Since previous studies indicated that they form a 1:1 complex, this molar ratio indicates that at least half of the CaVP is free or involved in interactions other than with CaVPT.

Although there is an array of observations that sarcoplasmic Ca^{2+}-binding proteins are genuinely sarcoplasmic (Wnuk et al. 1982), this study indicates that a portion of SCP is localized at the site of the isotropic band in muscle, a region rich in sarcoplasmic reticulum and in glycogen. A similar localization was reported for the sarcoplasmic Ca^{2+}-binding protein in crustacean muscle (Benzonana et al. 1977). The enrichment of at least two different SCPs at the isotropic band where glycogenolytic and glycolytic enzymes are preferentially located (Arnold and Pette 1970) may indicate that their physiological function is coordinated. A plausible hypothesis is that the physiologically important amounts of Mg^{2+} released from SCPs during prolonged stimulation of muscle (Cox 1990) stimulate the glycogenolytic and glycolytic enzymes. It is not yet clear if this particular distribution of SCPs is, as in the case of phosphagen kinases (Reddy et al. 1992) and glycolytic enzymes (Méjean et al. 1989), due to a specific interaction of SCPs with F-actin, or linked to the abundant glycogen particles in the I band. It is interesting in this respect that the specific staining of calmodulin in the isotropic band of gastrocnemius muscle is greatly reduced upon digestion of glycogen with α-amylase (Harper et al. 1980).

In addition to a general contribution to the background, CaVP and CaVPT form well-defined lines or bands at the level of the Z and M lines. The isotropic bands of amphioxus muscle are very narrow (Zapf and Mohamed 1959), which suggests that the Z line staining may in fact represent staining of the I band. The location in the I band may thus be of a similar nature to that of SCPs and creatine kinase. The intensity of this staining is constant in different preparations, whereas that of the M line is more variable. This suggests that the

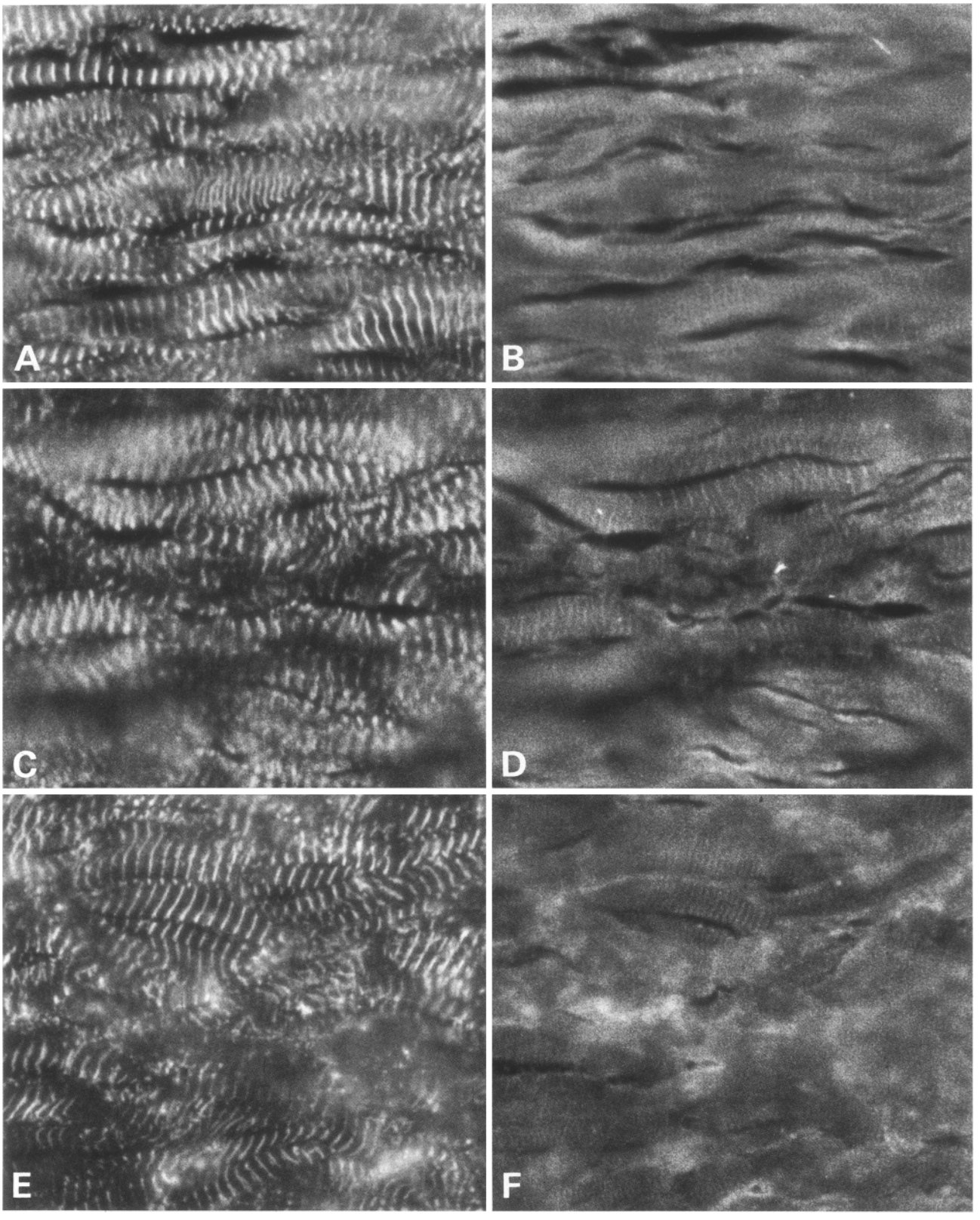

Fig. 5A–F. Photographs showing immunofluorescence of longitudinal sections of amphioxus muscle stained with antibodies against α-actinin (**A**, **C** and **E**) and the corresponding sections stained with anti-SCP (**B**), anti-CaVP (**D**) and anti-CaVPT (**F**). 1 cm = 8 μm

location of the CaVP/CaVPT pair at the M line is dynamic and perhaps dependent on the contraction or the presence of Ca^{2+}.

The question arises of which structure at the M line (or in the central part of the A band) is involved in the particular localization of the CaVP/CaVPT protein pair. The answer cannot yet be provided but a clue is the fact that CaVPT contains two copies of a sequence motif called the IgII domain, which is also present 6 times in C-protein and over 30 times in the tail region of titin. Both proteins strongly interact with myosin rods and with each other (Fürst et al. 1992; Labeit et al. 1992). If the IgII domains are instrumental in mutual associations between myosin, titin and C-protein, it can be speculated that other proteins containing such motifs, such as CaVPT, interfere with these associations. This interaction would then be more sophisticated if the interference is modulated by Ca^{2+} and CaVP.

A further question arises of whether the CaVP/CaVPT system shows a very limited distribution in the animal kingdom, such as the very specific myosin-linked regulation of muscular contraction in molluscs (Lehman and Szent-Györgyi 1975), or whether CaVP/CaVPT persisted during evolution up to the higher vertebrates. Using the polyclonal antibodies described in this study in conjunction with Western analyses, we could detect neither CaVP nor CaVPT in different rat tissues (unpublished results). Thus either this protein pair is absent from higher vertebrates or its counterpart in rat does not cross-react with our antibodies. Screenings in phyla closer to the cephalochordates are in progress to solve the question of the ubiquity of CaVP/CaVPT.

Acknowledgements. The authors thank I. Durussel for expert technical assistance and Dr. G. Benzonana for advice with antibodies. This work was supported by the Swiss National Science Foundation Grant nos. 31-28637.90 and 31-34062.92. Figure 1 was kindly provided by Prof. H. Huggel, Department of Zoology, Geneva.

References

Arnold H, Pette D (1970) Binding of aldolase and triosephosphate dehydrogenase to F-actin and modification of catalytic properties of aldolase. Eur J Biochem 15:360–366

Benian GM, Kiff JE, Neckelmann N, Moerman DG, Waterston RH (1989) Sequence of an unusually large protein implicated in regulation of myosin activity in *C. elegans*. Nature 342:45–50

Benzonana G, Wnuk W, Cox JA, Gabbiani G (1977) Cellular distribution of sarcoplasmic calcium-binding proteins by immunofluorescence. Histochemistry 51:335–341

Cox JA (1986) Isolation and characterization of a new M_r 18000 protein with calcium vector properties in Amphioxus muscle and identification of its endogenous target protein. J Biol Chem 261:13173–13178

Cox JA (1990) Unique calcium-binding proteins in invertebrates. In: Dedman JR, Smith VL (eds) Stimulus-response coupling: the role of intracellular calcium. CRC Press, Boca Raton, Ann Arbor, Boston, pp 67–72

Cox JA, Comte M, Stein EA (1981) Calmodulin-free skeletal-muscle troponin C prepared in the absence of urea. Biochem J 195:205–211

Cox JA, Alard P, Schaad O (1990) A predicted structure of amphioxus calcium vector protein from comparative molecular modeling with calmodulin and troponin C. Prot Engineering 4:23–32

Einheber S, Fischman DA (1990) Isolation and characterization of a cDNA clone encoding avian skeletal muscle C-protein: an intracellular member of the immunoglobulin superfamily. Proc Natl Acad Sci USA 87:2157–2161

Espreafico EM, Cheney RE, Matteoli M, Nascimento AC, De Camilli PV, Larson RE, Mooseker MS (1992) Primary structure and cellular localization of chicken brain myosin-V (p190), an unconventional myosin with calmodulin light chains. J Cell Biol 119:1541–1557

Flood PR (1975) Fine structure of the notochord of amphioxus. Symp Zool Soc Lond 36:81–104

Fürst DO, Vinkemeier U, Weber K (1992) Mammalian skeletal muscle C-protein: purification from bovine muscle, binding to titin and characterization of a full-length human cDNA. J Cell Sci 102:769–778

Gillespie PG, Hudspeth AJ (1991) Chemiluminescence detection of proteins from single cells. Proc Natl Acad Sci USA 88:2563–2567

Gopalakrishna R, Anderson WB (1982) Ca^{2+}-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-Sepharose affinity chromatography. Biochem Biophys Res Commun 104:830–836

Guthrie DM (1975) The physiology and structure of the nervous system of amphioxus (the lancelet) *Branchiostoma lanceolatum* Pallas. Symp Zool Soc Lond 36:43–80

Harlow E, Lane D (1988) Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Harper JF, Cheung WY, Wallace TW, Levine SN, Steiner AL (1980) Immunocytochemical localization of calmodulin in rat tissues. In: Cheung WY (ed) Calcium and cell function, vol I. Academic Press, New York, pp 273–290

Ito M, Dabrowska R, Guerriero V, Hartshorne DJ (1989) Identification in turkey gizzard of an acidic protein related to the C-terminal portion of smooth muscle myosin light chain kinase. J Biol Chem 264:13971–13974

Kobayashi T, Takagi T, Konishi K, Cox JA (1987) The primary structure of a new M_r 18000 calcium vector protein from Amphioxus. J Biol Chem 262:2613–2623

Kohler L, Cox JA, Stein EA (1979) Sarcoplasmic calcium-binding proteins in protochordate and cyclostome muscle. Mol Cell Biochem 20:85–93

Labeit S, Barlow DP, Gautel M, Gibson T, Hsieh C-L, Francke U, Leonard K, Wardale J, Whiting A, Trinick J (1990) A regular pattern of two types of 100-residue motif in the sequence of titin. Nature 345:273–276

Labeit S, Gautel M, Lakey A, Trinick J (1992) Towards a molecular understanding of titin. EMBO J 11:1711–1716

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lehman W, Szent-Györgyi AG (1975) Regulation of muscular contraction: Distribution of actin control and myosin control in the animal kingdom. J Gen Physiol 66:1–30

Lennette DA (1978) An improved mounting medium for immunofluorescence microscopy. Am J Clin Pathol 69:647–648

Méjean C, Pons F, Benyamin Y, Roustan C (1989) Antigenic probes locate binding sites for the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, aldolase and phosphofructokinase on the actin monomer in microfilaments. Biochem J 264:671–677

Moncrief ND, Kretsinger RH, Goodman M (1990) Evolution of EF-hand calcium-modulated proteins. I. Relationships based on amino acid sequences. J Mol Evol 30:522–562

Nakayama S, Moncrief ND, Kretsinger RH (1992) Evolution of EF-hand calcium-modulated proteins. II. Domains of several subfamilies have diverse evolutionary histories. J Mol Evol 34:416–448

Olson NJ, Pearson RB, Needleman DS, Hurwitz MY, Kemp BE, Means AR (1990) Regulatory and structural motifs of chicken gizzard myosin light chain kinase. Proc Natl Acad Sci USA 87:2284–2288

Reddy SRR, Houmeida A, Benyamin Y, Roustan C (1992) Interaction in vitro of scallop muscle arginine kinase with filamentous actin. Eur J Biochem 206:251–257

Surolia A, Pain D, Khan MI (1982) Protein A: nature's universal anti-antibody. Trends Biol Sci 7:74–76

Takagi T, Cox JA (1990a) Amino acid sequences of four isoforms of amphioxus sarcoplasmic calcium-binding proteins. Eur J Biochem 192:387–399

Takagi T, Cox JA (1990b) Primary structure of CaVPT, the target of calcium vector protein of amphioxus. J Biol Chem 265:19721–19727

Takagi T, Konishi K, Cox JA (1986) The amino acid sequence of two sarcoplasmic calcium-binding proteins from the protochordate Amphioxus. Biochemistry 25:3585–3592

Takagi T, Valette-Talbi L, Cox JA (1992) Primary structure of three minor isoforms of amphioxus sarcoplasmic calcium-binding protein. FEBS Lett 302:159–160

Thorpe A, Thorndyke CM (1975) The endostyle in relation to iodine binding. Symp Zool Soc Lond 36:159–178

Welsch U (1975) The fine structure of the pharnyx, cyrtopodocytes and digestive caecum of amphioxus. Symp Zool Soc Lond 36:17–42

Wnuk W, Cox JA, Stein EA (1982) Parvalbumin and other soluble sarcoplasmic Ca-binding proteins. In: Cheung WY (ed) Calcium and cell function, vol II. Academic Press, New York, pp 243–278

Wylie DC, Vanaman TC (1988) Structure and evolution of the calmodulin family of calcium regulatory proteins. In: Cohen P, Klee CB (eds) Calmodulin. Elsevier, Amsterdam New York Oxford, pp 1–15

Zapf K, Mohamed AAA (1959) Zur submikroskopischen Morphologie und Funktion der quergestreiften Muskulatur des *Branchiostoma lanceolatum* (Amphioxus). Acta Biol Med Ger 2:508–519