Myophilin of *Echinococcus granulosus*: isoforms and phosphorylation by protein kinase C

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SUMMARY

Myophilin is a muscle-associated antigen of the taeniid cestode *Echinococcus granulosus*. This protein shows a high amino acid sequence homology with calponins and calponin-like proteins, which are proposed to be associated with the regulation of smooth muscle contraction. In order to provide supportive evidence for a relationship between these proteins, we characterized myophilin using electrophoretic, biochemical and molecular biological approaches. Two-dimensional protein electrophoretic separation of *E. granulosus* larval proteins defined 4 isoelectric isoforms of myophilin (α, β, γ and δ), which appeared to be a consequence of post-translational modification of a single gene product. It was also demonstrated biochemically that *E. granulosus* myophilin undergoes specific phosphorylation *in vitro* by protein kinase C (PKC). Finally, myophilin homologues were identified in extracts of *Taenia hydatigena* and *T. ovis* by immunoblot. A partial cDNA of the closely related species, *E. multilocularis*, was isolated by cloning procedures and showed 99% homology with the *E. granulosus* myophilin gene. The similarities of *E. granulosus* myophilin with calponins in their tissue localization, protein isoform patterns, ability to be phosphorylated *in vitro* by PKC, and the relatively conserved nature of the protein among related parasites suggest that myophilin may be associated with smooth muscle contraction.

Key words: Taeniidae, *Echinococcus*, myophilin, calponin, protein kinase C.

INTRODUCTION

Infection of humans with the larval stage of the hydatid tapeworm, *Echinococcus granulosus*, is of major public health significance in many countries. Although this parasite has been the subject of much research over the years (Thompson & Lymbery, 1995), there remains a paucity of information on fundamental aspects of its physiology, biochemistry and molecular biology (McManus & Bryant, 1995).

Recently, myophilin, an antigenic muscle-associated protein of *E. granulosus* was cloned (Martin *et al*. 1995, 1996). The amino acid sequence of myophilin shows a high (40–55%) homology to proteins known as 'calponins' and 'calponin-like proteins' (Pearlstone *et al*. 1987; Ayme-Southgate *et al*. 1989; Takahashi & Nadal-Ginard, 1991; Nishida, Kitami & Hiwada, 1993; Prinijha *et al*. 1994). Although the biological role(s) of calponin-like proteins is not fully defined, there is evidence that calponins are associated with smooth muscle contraction (Pearlstone *et al*. 1987; Nishida *et al*. 1991; Prinijha *et al*. 1994; Kobayashi, Kubota & Hidaka, 1994). It has been shown that calponins are involved in the regulation of muscle contraction by mechanisms of reversible phosphorylation mediated by protein kinase C (PKC) (Winder & Walsh, 1990; Winder, Sutherland & Walsh, 1991; Winder *et al*. 1993), by their interaction with calcium–calmodulin (Takahashi, Hiwada & Kokubu, 1986) and by their ability to bind actin and tropomyosin (Mezgueldi *et al*. 1992, 1995; Castresana & Saraste, 1995). This latter characteristic has recently been associated with an N-terminal domain (calponin-homology (CH) domain) of the calponin amino acid sequence (Castresana & Saraste, 1995). Myophilin shows 35–53% homology in this domain (164 amino acids) with a range of calponins and calponin-like proteins, including vertebrate SM22 and *Drosophila melanogaster* mp20.

Based on this information and the fact that there are significant structural similarities between smooth musculature of cestodes and vertebrates (Lumsden & Byram, 1967; Ward, Allen & McKerr, 1986; Ward, McKerr & Allen, 1986), it could be hypothesized that myophilin may play a role in the regulation of muscle contraction in cestodes, similar

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to that proposed for some calponins of vertebrates. In this study, we have analysed electrophoretic and biochemical features of the native and recombinant forms of the *E. granulosus* myophilin protein in order to provide evidence in support of this hypothesis.

MATERIALS AND METHODS

Parasites and antigens

Live larval stages (= protoscoleces) of *E. granulosus* were isolated from liver cysts from a naturally infected kangaroo in Australia. Adult worms of *E. multilocularis* and *Taenia* spp. were collected from naturally or experimentally infected canids. Parasites were washed extensively in phosphate-buffered saline (PBS), pH 7·4, and snap frozen in liquid nitrogen.

Protein electrophoresis

Parasite antigens were prepared for electrophoretic separation as follows. A packed volume (200 μl) of parasite material was boiled and vortexed in 500 μl of sodium dodecylsulfate (SDS) sample buffer (30 % glycerol, 2 % SDS, 10 mM β-mercaptoethanol, 25 mM Tris–HCl, pH 6·5) for 5 min until suspended, centrifuged and stored in aliquots of 100 μl at −70 °C. One-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to standard procedures (Laemmli, 1970). Ten μl of antigen (approximately 5 μg) was loaded into each well. Two-dimensional gel electrophoresis (IEF/SDS–PAGE) was performed according to the method of O'Farrell (1975). A volume of 100 μl of antigen (approximately 50 μg) was subjected to isoelectric focusing in the first-dimension using rod gels (1·5 mm diameter) consisting of 9·5 M urea, 2·7 % acrylamide, 0·14 % *bis*-acrylamide, 2 % Nonidet P-40, 5 % β-mercaptoethanol, 2 % Bio-Lyte (BioRad) ampholytes, covering pH ranges 5–7 (1·6 %) and 3–10 (0·4 %), run at 20000 Vh. Electrophoretic separation of proteins in the second dimension was performed in the ProteanIIxi system (BioRad) using 16·5 % polyacrylamide gels. Proteins were stained with Coomassie Brilliant Blue R-250 (BioRad).

Immunoblot

Separated proteins were transferred from electrophoretic gels to nitrocellulose using the SemiDry™ electrotransfer system (BioRad). Membranes were probed with a hyperimmune rabbit antiserum (α-10P1-GST; 1/5000 dilution) raised against recombinant myophilin as described previously (Martin *et al*. 1995). This antiserum showed specific reactivity with *Echinococcus* myophilin (Martin *et al*. 1995).

Northern blot

E. granulosus protoscoleces were homogenized in liquid nitrogen, and total RNA was extracted using guanidine thiocyanate solution (Chomczynski & Sacchi, 1987). RNA (2–10 μg) was electrophoresed in 1 % agarose–5 % formaldehyde gels and blotted onto positively charged nylon membranes (Boehringer-Mannheim) in 20 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7·0). After rinsing with water and UV cross-linking (Spectronics UV cross-linker 1500), blots were pre-hybridized at 50 °C for 3 h in SDS–formamide buffer (7 % SDS, 20 mM maleic acid, pH 7·5, 50 % formamide, 2 % blocking reagent, 5 × SSC, 0·1 % lauroylsarcosine). Hybridization was carried out at 50 °C in the same solution with the digoxigenin-11-dUTP-labelled DNA probe fRM3/RM2 (Martin *et al*. 1995). Stringent washes were carried out at 68 °C in 0·1 × SSC/0·1 % SDS, and detection of the probe was performed according to the manufacturer's protocol (Boehringer-Mannheim).

Expression of recombinant myophilin and myophilin ΔC

A 5′ *Bam*HI site in frame with the open reading frame of both myophilin and the polyhistidine tag of pQE-30 (Qiagen), was introduced into the myophilin cDNA by PCR. Plasmid pGEM5-Eg/myophilin, containing the full-length cDNA, was used as PCR template with 5′-myophilin primer *Bam*HI-RM8: 5′-CGGGATCCCATGTCGAACGTTC-3′ and 3′-vector primer SP6. Amplification was performed on a DNA Thermal Cycler (Perkin-Elmer) in a total volume of 50 μl, containing 40 μM each of the 4 dNTPs, 2·5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9·0) and 1·25 U *Taq* DNA polymerase (Promega). Reactions were run for 30 cycles using the following conditions: 94 °C, 30 sec; 50 °C, 30 sec; 72 °C, 1 min, followed by 1 extension cycle at 72 °C for 7 min. The PCR product was purified using silica-resin (Promega), digested with *Bam*HI/*Sph*I and subcloned into the expression vector pQE-30. To construct the C-terminal deletion clone pQE-Eg/myophilinΔ*C*, a 420 bp *Pst*I restriction fragment, derived from the *Pst*I sites present in the myophilin cDNA (bp 569) and the adjacent multiple cloning site of the vector, were deleted from pQE-EG/myophilin. Plasmids pQE-Eg/myophilin and pQE-Eg/myophilinΔC were transformed to SG13009[pREP4] *E. coli* cells (Qiagen). Cultures were induced with 1 mM IPTG and screened for expression by SDS–PAGE. Recombinant myophilin (aa 1–190) and myophilinΔC (aa 1–178), expressed with a polyhistidine (6 ×) tag at their N-termini, were affinity purified over a Ni–NTA resin and eluted with an imidazole gradient (0–0·5 M) according to the manufacturer's protocol (Qiagen). Recom-

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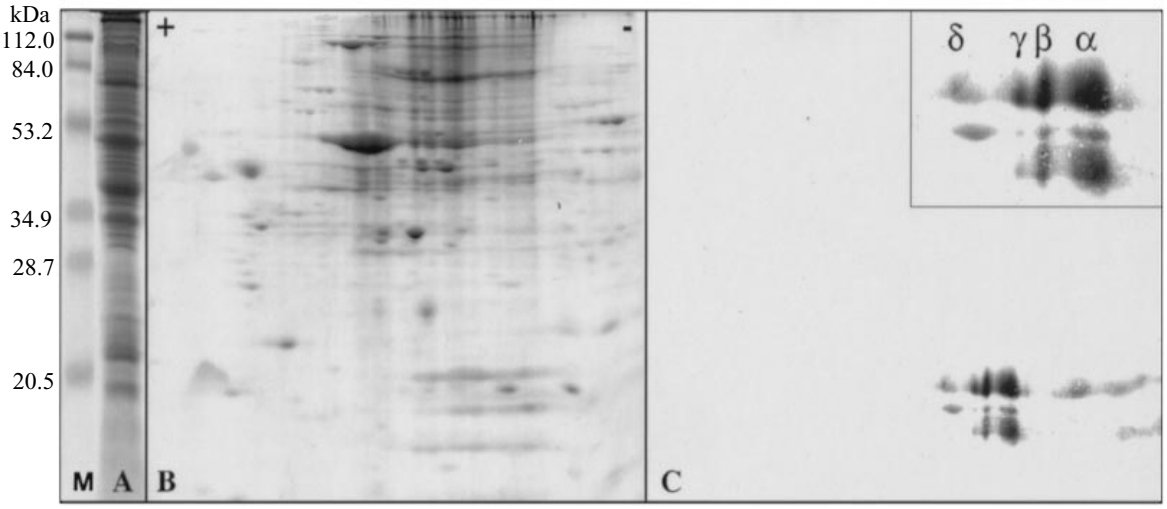

Fig. 1. One- and two-dimensional electrophoretic analyses of *Echinococcus granulosus* protoscolex proteins. (A) Coomassie-stained SDS–PAGE (16·5 %) gel (B) Coomassie-stained IEF/SDS–PAGE (16·5 %) gel (C) Identical panel to (B) examined by immunoblot using myophilin antiserum. The inset of (C) is an enlargement of the area of specific recognition. α, β, γ and δ indicate isoforms of myophilin, from basic to acidic. (M) Molecular weight standard separated in an SDS–PAGE (16·5 %) gel.

binant proteins were confirmed to be myophilin and myophilinΔC by immunoblot using myophilin antiserum.

In vitro *phosphorylation of myophilin*

Protein kinase C was purified from rat brain as described previously (Phillips, Croatto & Hamilton, 1992). In brief, rat brain was homogenized, sonicated and subjected to ultracentrifugation (60000 ***g***) for 1 h at 4 °C, and the membrane-containing supernatant retained and sonicated. PKC was semi-purified by anion-exchange using a DEAE–Sepharose (Pharmacia) column and an NaCl gradient. Five μg of either myophilin or myophilinΔC were incubated with 20 mg of PKC extract for 20 min at 30 °C in 40 ml of kinase assay buffer containing 0·6 mM $CaCl_2$, 10 mM $MgCl_2$, 40 mM ATP, 2 mM EGTA, 1 mM phenylmethyl-sulfonyl fluoride, 50 mg/ml leupeptin, 0·35 % (v/v) β-2 mercaptoethanol, 10 mg/ml phosphatidyl serine, 10 mg/ml, 1,2-diolein, 0·2 mM NaF, 0·1 mM sodium orthovanadate and 27·8 mCi of [γ-^{32}P] ATP/ml in 50 mM Tris–HCl, pH 7·5. Reactions were stopped by adding SDS-sample buffer. Samples were boiled for 5 min and subjected to SDS–PAGE. Gels were stained with Coomassie blue, dried and auto-radiographed. Quantitation of Coomassie blue-stained proteins and incorporation of [^{32}P] by PKC was performed by densitometry on gels and auto-radiographs with ImageQuant™ software (Molecular Dynamics).

E. multilocularis *gene library and screening*

A cDNA library was constructed from mRNA derived from adult *E. multilocularis* worms. Poly A$^+$RNA was purified from 500 μg total RNA using the polyATract™ mRNA isolation system (Promega). Five μg polyA$^+$ RNA was used for cDNA synthesis and construction of a unidirectional library in λZAP II using the ZAP–cDNA synthesis kit (Stratagene) according to the manufacturer's protocol. The library contained 6×10^5 primary clones, of which approximately 80 % represented recombinants. A PCR-based strategy (Martin *et al.* 1995) was employed to screen the *E. multilocularis* cDNA library. In brief, partial cDNAs were amplified from the library using the 5′-myophilin-specific primer RM1 (5′-AGACATACTGAAGGATGGCACC-3′) and the 3′-vector primer T7, or with the 5′-vector primer T3 and the 3′-myophilin-specific primer RM6 (5′-GCTGCTTGCCCATATTGATACC-3′). The specificity of the amplified fragments was confirmed by Southern blot hybridization with fRM3/RM2.

DNA sequencing and analysis

DNA sequences (both strands) of plasmid inserts and PCR fragments were determined by cycle-sequencing using the *f*mol™ cycle-sequencing kit (Promega) using appropriate PCR primers. Sequences were aligned and analysed using the MacVector™ (IBI) and AssemblyLign™ (IBI) programs.

RESULTS

Characterization of native E. granulosus *myophilin*

E. granulosus protoscolex antigen was resolved by SDS–PAGE (Fig. 1 A) and IEF/SDS–PAGE (Fig. 1 B). A two-dimensional gel was blotted onto nitro-cellulose membrane and probed with myophilin

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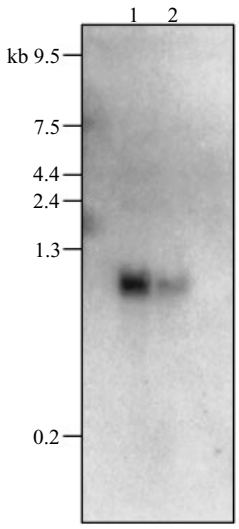

Fig. 2. Northern blot of *Echinococcus granulosus* protoscolex RNA. Total RNA 7·5 μg (lane 1) and 2·5 μg (lane 2) probed with the myophilin specific DNA probe fRM3/RM2.

antiserum (Fig. 1 C). Myophilin antiserum repeatably detected 4 isoelectric variants (= isoforms) of the same apparent molecular weight (approximately 22 kDa) in the basic region of the gel (Fig. 1 C). The isoforms were designated α, β, γ and δ in order of decreasing basicity (inset Fig. 1 C). The difference in intensity between the isoforms indicated a difference in quantity, with the most basic (α) variant being in the highest and the most acidic (δ) isoform in the lowest abundance, respectively. Proteins detected at approximately 19 kDa and 17 kDa showed a similar isoform pattern as the 22 kDa fraction. Interestingly, the most acidic isoform (δ) was absent in the 17 kDa fraction. To examine whether the proteins of different molecular weight were derived from different mRNAs, a Northern blot of total RNA from *E. granulosus* protoscoleces was performed using fRM3/RM2 as a probe. A single band of approximately 1 kb, corresponding to the size of the myophilin cDNA, was detected (Fig. 2).

Phosphorylation of recombinant E. granulosus *myophilin*

The amino acid sequence of recombinant *E. granulosus* myophilin revealed 2 putative PKC sites (Ser-64 and Ser-171) (Martin *et al.* 1995). In order to determine whether these represented true phosphorylation sites, myophilin and myophilinΔC (from which PKC site Ser-171 was deleted) were tested with PKC *in vitro*. The ratio of ^{32}P incorporation for myophilin/myophilinΔC, as assessed by densitometry was 1·96 : 1, in accordance with the expected ratio of 2 : 1 for specific phosphorylation (Fig. 3). Phosphorylation did not change the migration behaviour of myophilin or myophilinΔC on SDS–PAGE gels.

Expression of myophilin in related taeniid cestodes

Experiments were conducted to evaluate whether myophilin homologues existed in related taeniid cestodes. In a first step, an immunoblot analysis of antigens derived from *E. granulosus* and *Taenia* spp. was performed using myophilin antiserum (Fig. 4). In contrast to a previous study, where a single broad myophilin band was detected in *E. granulosus* using SDS–PAGE 13 % gels (Martin *et al.* 1995), the better separation of low molecular weight proteins achieved on large 16·5 % gels allowed the reproducible resolution of 3 bands (22, 19 and 17 kDa) (Fig. 4, lane 1). The bands of lower molecular weight were assumed to be proteolytic degradation products of the 22 kDa protein and, although present in the antigen preparation, could not be resolved on the 13 % mini-gel system. Bands of the same molecular

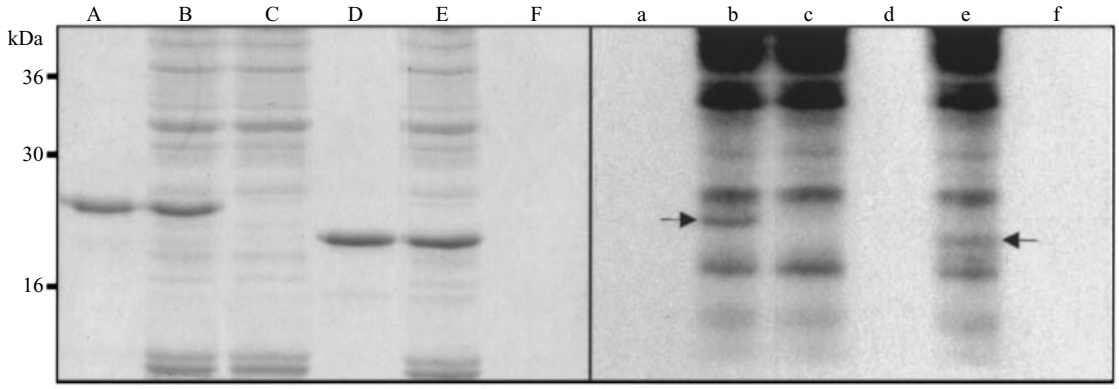

Fig. 3. PKC-catalysed phosphorylation of recombinant myophilin and myophilinΔC. Lanes A–F (left) Coomassie stained SDS–PAGE (13 %) gel, lanes a–f (right) autoradiograph of the same gel. Lanes A/a, recombinant myophilin; lanes B/b, recombinant myophilin and PKC extract from rat brain; lanes C/c, PKC extract from rat brain; lanes D/d, recombinant myophilinΔC; lanes E/e, myophilinΔC and PKC extract from rat brain; lanes F/f, PKC reaction buffer with co-factors. Arrows indicate the location of ^{32}P-labelled recombinant myophilin and myophilinΔC after incubation with PKC.

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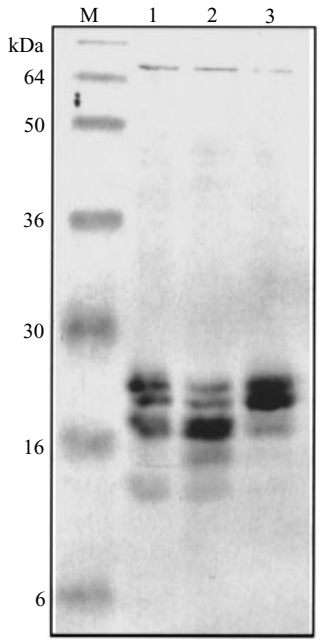

Fig. 4. Detection of myophilin in taeniid cestode species by immunoblot. Antigens of *Echinococcus granulosus* protoscoleces (lane 1) and worms of *Taenia hydatigena* (lane 2) and *Taenia ovis* (lane 3) were separated in a SDS–PAGE (16·5 %) gel, blotted and probed with myophilin antiserum.

weights were also identified in antigens from *Taenia* species (Fig. 4, lanes 2 and 3), although there was a difference in the intensity of individual bands. Subsequently, *E. multilocularis*, a closely-related parasite to *E. granulosus*, was examined for the presence of a myophilin gene. PCR screening of the *E. multilocularis* cDNA library amplified an 800 bp fragment with primer set RM1/T7, and a 580 bp PCR fragment was amplified with primer set T3/RM6 (results not shown). The overlapping nucleotide (nt) sequences of the two fragments revealed 397 bp of coding sequence (accession number X95464) with 98·9 % identity to the *E. granulosus* myophilin cDNA sequence (nt positions 234–631). At nt position 360, relative to the *E. granulosus* cDNA sequence, a G was replaced by a T, resulting in a substitution of Gly with Val. At nt positions 390, 420 and 594, a C was replaced by a T, without resulting in a change of the amino acid sequence.

DISCUSSION

In this study, we have identified multiple isoforms of myophilin in *E. granulosus* protoscoleces by two-dimensional gel electrophoresis. The isoforms α, β, γ and δ were of similar molecular weight (22 kDa) but differed in their isoelectric points (pI) and intensities. Two fractions of lower molecular weight (17 kDa and 19 kDa) were also detected in one- and two-dimensional electrophoretic gels and confirmed to be myophilin-specific on immunoblot using myophilin antiserum. It was initially presumed that the different sized fractions were a result of post-translational glycosylation of myophilin. However, treatment of parasite extract with *N*-glycosidase F did not alter the banding pattern in immunoblot, thereby excluding *N*-glycosylation as a possible cause (data not shown). The presence of a single myophilin mRNA transcript in protoscoleces indicated that the 17 and 19 kDa fractions were probably not a consequence of alternative splicing, but rather of proteolytic degradation of the 22 kDa protein. This proposal was supported by computer analyses which predicted that the two low molecular weight fragments were the result of proteolytic digestion at N- and C-terminal regions, most exposed on the surface of *E. granulosus* myophilin (Martin *et al*. 1995).

In spite of differences in the pI and molecular weights of protein fractions, the isoform pattern of myophilin is similar to that described for the vertebrate proteins calponin (Gimona *et al*. 1992; Strasser *et al*. 1993; Winder *et al*. 1993) and SM22 (Pearlstone *et al*. 1987; Lees-Miller, Heeley & Smillie, 1987; Lees-Miller *et al*. 1987). Such isoform patterns appear to be associated with the functional capacity of the muscle cell and development during smooth muscle differentiation as a result of post-translational modification(s) (Gimona *et al*. 1992). However, the exact nature of the post-translational modification(s) in calponin and SM22 remain(s) unknown (Lees-Miller, Heeley & Smillie, 1987; Winder *et al*. 1993). We investigated whether the more acidic (β, γ and δ) isoforms of myophilin represented multiple phosphorylation stages of the major α-isoform by incubation of parasite antigen with calf intestine alkaline phosphatase. No changes were observed in the isoform pattern of myophilin (results not shown), supporting the data reported for both calponin and SM22, where phosphatase treatment or anti-phosphoserine antibodies could not detect differential phosphorylation of the isoforms (Lees-Miller *et al*. 1987; Barany, Rokolya & Barany, 1991; Gimona *et al*. 1992). It was interesting that in the 17 kDa fragment of myophilin, the most acidic δ-isoform, was not detectable on two-dimensional gels. This observation may indicate that post-translational modifications on the extreme N- and/or C-terminus of myophilin, which is thought to be truncated in the 17 kDa fraction, may be responsible for the acidic pI.

Although it appears that phosphorylation is not responsible for generating calponin and myophilin isoforms, several studies have described that calponin is phosphorylated *in vivo* (Winder, Pato & Walsh, 1992; Nishida *et al*. 1991; Gerthoffer & Pohl, 1994; Mino *et al*. 1995). Phosphorylation–dephosphorylation of calponin by PKC is considered important in regulating calponin function (Winder & Walsh, 1990; Winder *et al*. 1993). The similarities

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between myophilin and calponin in amino acid sequence, tissue localization and isoform expression led us to investigate whether myophilin could be phosphorylated by PKC *in vitro*. We demonstrated that differential phosphorylation of recombinant myophilin and the C-terminal deletion protein, myophilinΔC, could be achieved. One phosphate molecule was incorporated at site 'Ser-64' (amino acid region 1–167) and a second at site 'Ser-171′ (C-terminal 168–190 region). The 168–190 region of myophilin shows a high homology (52–55 %) to the C-terminal repeat of calponins, which represents the major phosphorylation site of calponin (Winder *et al*. 1993) and is considered a regulatory domain of the protein (Mezgueldi *et al*. 1992, 1995). To our knowledge, this is the first report of a calponin-like protein (i.e. myophilin) with one C-terminal calponin-repeat as a substrate for PKC *in vitro*. In order to determine the physiological relevance of myophilin phosphorylation, further work is required to examine phosphorylation *in vivo* and to identify *Echinococcus*-specific myophilin kinase(s) and phosphatase(s).

The amino acid sequences of myophilin, SM22 and calponin contain regions with homology to the EF-hand motif of 'calcium binding proteins' (Martin *et al*. 1995). It was anticipated that myophilin may bind calcium directly. However, experiments demonstrated that the protein did not bind physiological concentrations of ^{45}Ca (data not shown). This result is in line with some other studies of proteins possessing EF-hand motifs. For example, no calcium binding was demonstrated for SM22 proteins (Takahashi, Hiwada & Kokubu, 1987; Lees-Miller *et al*. 1987), while a low Ca^{2+} affinity (K_d 7 μM) was reported for calponin (Takahashi, Hiwada & Kokubu, 1986). Based on this information, the ability of myophilin and calponins to bind Ca^{2+} remains controversial. However, it is possible that the mechanism for Ca^{2+} binding is indirect (via for example calmodulin) rather than direct (Wills, McCubbin & Kay, 1993). This aspect clearly warrants investigation. Finally, the presence of a highly conserved myophilin gene in *E. multilocularis* and the detection of myophilin homologues in other taeniids examined herein suggest that the myophilin protein plays an important physiological role in the smooth muscle of tapeworms. The structural and biochemical similarities of myophilin to calponin suggest that there is a functional relationship between the proteins. Future research should identify *E. granulosus* molecules that interact directly with myophilin, and determine the nature of this interaction, in order to elucidate the precise role(s) of myophilin in the smooth musculature of cestodes.

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