

# Mammalian calponin

## Identification and expression of genetic variants

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Calponin is a smooth muscle specific, actin-, tropomyosin- and calmodulin-binding protein thought to be involved in some way in the regulation or modulation of contraction. Here we describe the cloning and bacterial expression of two calponin species from murine and porcine smooth muscle tissues. Primary and secondary structural analyses of the deduced amino acid sequences revealed a high degree of homology to avian calponin with the exception of a short and variable C-terminal segment. The sequence data demonstrate that the two mammalian calponin variants do not arise via alternative splicing but are encoded by different genes

Smooth muscle; Calponin; Actin-binding protein; Bacterial expression

### 1. INTRODUCTION

Calponin is a basic 34-kDa protein first isolated from chicken gizzard and bovine aorta [1,2] that is specifically expressed in smooth muscles [3]. It is a heat-stable, calmodulin-, actin- and tropomyosin-binding protein [1–4] that is capable of inhibiting in vitro the actin-activated MgATPase of myosin in a dose-dependent manner [5–8]. From its in vitro inhibitory properties calponin has been attributed with a regulatory role in smooth muscle contraction [5,7]. Further support for this idea comes from the demonstration that calponin induces conformational changes in F-actin [9], can arrest movement of actin filaments in motility assays [10] and may itself undergo conformational changes in vivo that are associated with the contractile process [11].

However, current information on the mode of action and regulation of calponin on the smooth muscle thin filament is both controversial and incomplete. In one series of studies [5,12] the reversible phosphorylation of calponin was claimed to regulate this protein's activity, but these findings were inconsistent with the inability to detect any phosphorylation of calponin in vivo [13,14].

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*Abbreviations* PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; pI, isoelectric point.

The nucleotide sequences from which the amino acid sequences reported in this paper were deduced have been deposited in the EMBL/GenBank Data Libraries and assigned the accession numbers Z19542 (mouse  $h_1$ -calponin), Z19538 (pig  $h_1$ -calponin), Z19543 (mouse  $h_2$ -calponin) and Z19539 (pig  $h_2$ -calponin).

Likewise, the form of the regulatory complex associated with the thin filament, for example, whether it includes caldesmon and calponin together or as separate entities, is unclear [15]. More attention to the specific role of calponin in smooth muscle regulation is thus called for.

Earlier studies have centred on calponin isolated from avian gizzard, and the complete sequence has been established for the chicken protein [16]. From the sequence data as well as from studies on proteolytic fragments [4] the general domains of the molecule involved in calmodulin- and actin-binding have also been identified [17]. But detailed functional studies of the subdomains of calponin have not yet been performed. As a first step towards mapping the functional parts of the molecule we have cloned calponin from two mammalian smooth muscles. In so doing we have also identified a second and new calponin variant which, from sequence analysis, is deduced to arise from a separate gene.

### 2. MATERIALS AND METHODS

#### 2.1. Materials and general techniques

Unless stated otherwise all DNA manipulations were carried out according to standard protocols [18]. *Escherichia coli* DH5 $\alpha$  (Gibco BRL) was used as a standard host in all cloning experiments, *E. coli* BL21(DE3) [19] served as a host for expressing the calponin variants. Bacteria were grown in TB medium (2.7% (w/v) yeast extract (Gibco BRL); 1.3% (w/v) Peptone 140 (Gibco BRL); 17 mM KH<sub>2</sub>PO<sub>4</sub>; 72 mM Na<sub>2</sub>HPO<sub>4</sub>). Selection media were supplemented with 0.1 g/liter ampicillin.

Vectors used for cloning and sequencing were pBluescript plasmids (Stratagene); the expression vector was pMW172 [20]. *E. coli* BL21(DE3) and plasmid pMW172 were a gift of Dr. M. Way (MRC Laboratory of Molecular Biology, Cambridge, UK)

A  $\lambda$ gt11 cDNA library from mouse uterus was purchased from Clontech and a  $\lambda$ gt11 cDNA library from pig stomach was kindly provided by Prof F Wuytack (University of Leuven, Belgium).

Another pig stomach  $\lambda$ gt11 cDNA library was also constructed following a standard protocol (Promega Corporation) using *EcoRI*-adaptors,  $\lambda$ -arms and the  $\lambda$ -packaging extract from Promega Corporation.

All restriction and modifying enzymes were purchased from New England Biolabs, Boehringer Mannheim or Gibco BRL and were used according to the manufacturer's recommendations. A sequenase version 2.0 kit (United States Biochemical Corp.) was used for DNA sequencing. Hybridizations (screening of cDNA libraries, Northern blots) were carried out in the buffer according to Church and Gilbert [21] and the filters exposed to X-ray film (Kodak X-OMAT S or X-AR 5).

## 2.2. Isolation of poly(A)<sup>+</sup> RNA and Northern blotting

Total RNA from mouse uterus, pig aorta, pig stomach and pig uterus was extracted according to the method of Chirgwin et al. [22]

followed by urea/LiCl precipitation. Poly(A)<sup>+</sup> RNA was obtained by subsequent oligo(dT)-cellulose affinity chromatography (Pharmacia). For Northern blot analysis RNA was separated by gel electrophoresis on a 1% agarose gel containing 6% formaldehyde using 40 mM Bicine/NaOH (pH 8.3) as a running buffer. The gels were blotted overnight onto Hybond-N (Amersham) or nitrocellulose BA 85 (Schleicher and Schuell) membranes as described [18], and the transferred RNA was cross-linked by exposing the blots to UV light (312 nm) for 140 s (Hybond-N membranes) or by baking the filters for 90 min at 80°C in a vacuum oven (nitrocellulose membranes).

## 2.3. Amplification of a calponin-specific probe for screening cDNA libraries

Poly(A)<sup>+</sup> RNA from pig stomach was copied into double-stranded cDNA according to a standard protocol for one-tube cDNA synthesis (Bethesda Research Laboratories) with minor modifications. This DNA was subjected to a polymerase chain reaction (PCR) using the following degenerate primers: the upstream primer matched the amino acids 7–13 (-NRGPAYG-) of the avian calponin sequence [16], and

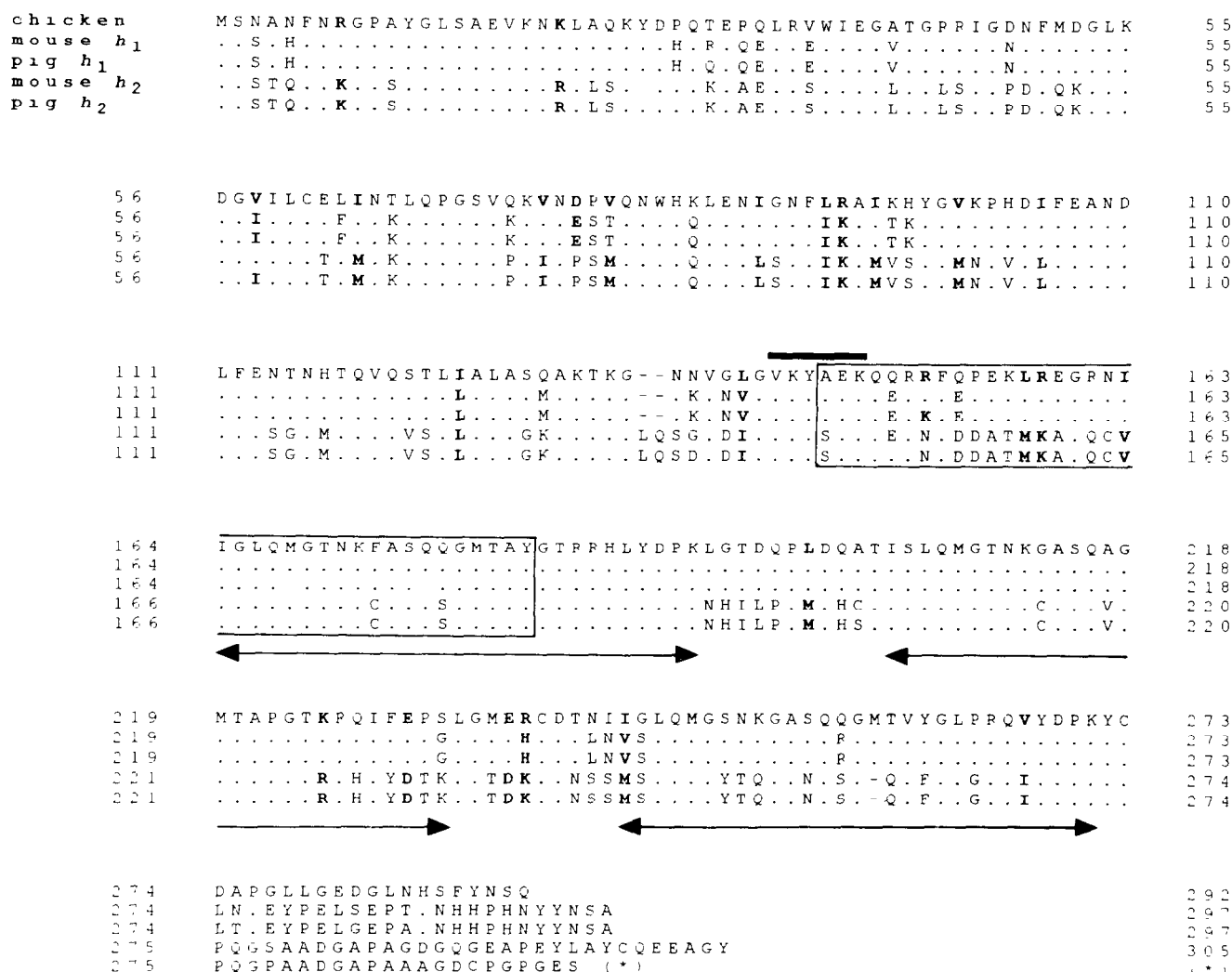


Fig. 1. Comparison of the amino acid sequences of mouse and pig calponin variants derived from the nucleotide sequences. The chicken calponin sequence [16] is shown in the first line. The asterisk (\*) at the end of the pig  $h_2$ -sequence indicates the missing C-terminus. Dots signify sequence identity to chicken calponin, and bold letters conservative exchanges. The boxed region highlights the putative actin-binding domain in chicken calponin as derived via limited proteolysis by Mezgueldi et al. [17], and the solid bar marks the position of a putative actin-binding sequence motif as suggested by the same authors. The sequences indicated by double-ended arrows represent the three quasi-repeats [16]. Numbering of amino acids is in accordance with chicken  $\alpha$ -calponin [16].

the downstream primer covered the sequence -ASQQ(A)GMT- corresponding to amino acid positions 174–180, 214–220 and 253–259. The amplified DNA fragment was cloned, sequenced and shown to correspond to the region between residues 7–220, numbered according to the avian sequence [16]. This fragment was used as a probe for screening  $\lambda$ gt11 cDNA libraries from mouse uterus and pig stomach.

#### 2.4. Expression of calmodulin in *E. coli*

The cDNAs coding for  $h_1$ - and  $h_2$ -calponin (the two variants identified; see section 3) from mouse uterus were cloned into the prokaryotic expression vector pMW172 [20]. In a two-step construction a *BalI*–*ApaI* fragment starting 5 bp upstream of the initiation codon of  $h_1$ -calponin and ending 3 bp downstream of the stop codon was inserted into pMW172 giving rise to plasmid MWCAL-1. Similarly, a  $h_2$ -calponin fragment starting at a *Bst*UI site 5 bp upstream of the initiating ATG codon and extending to a *SmaI* site approximately 360 bp downstream from the stop codon was cloned into pMW172 ( $\rightarrow$ MWCAL-2). Both constructs were checked by sequencing across the cloning sites which confirmed that no frame shifts or fusions to foreign sequences had occurred. Expression in *E. coli* BL21(DE3) was induced as described [20].

#### 2.5. Purification and electrophoresis of proteins

Purification of calponin from smooth muscle tissue has been reported previously [4]. SDS-polyacrylamide gel electrophoresis was carried out on 8–22% gradient acrylamide minislab gels according to Matsudaira and Burgess [23] in the buffer system of Laemmli [24]. Two-dimensional gel electrophoresis was performed according to O'Farrell [25] with some modifications [14]. Immunoblotting was carried out as described [14].

#### 2.6. Predictions of protein structure

Structural predictions of the calponin variants were made using the computer program UWGCG 7 with the algorithms according to Garnier–Osguthorpe–Robson [26] for secondary structure and according to Hopp and Woods [27] for surface location probability.

### 3. RESULTS

#### 3.1. Isolation and sequencing of cDNAs encoding calponin

Screening of  $\lambda$ gt11 cDNA libraries from mouse uterus and pig stomach with the PCR fragment described (see section 2) led to the isolation of two classes of cDNAs, according to restriction mapping. Several clones from each tissue and group were selected for partial nucleotide sequencing, and one of each was finally sequenced on both strands throughout the entire length. Comparison of the deduced amino acid sequences showed that one class of clones with an insert size of approximately 1,500 bp coded for a calponin species which is highly homologous to  $\alpha$ -calponin from chicken gizzard [16] whereas the second class (insert size approximately 1,800 bp) encoded a novel variant, different in sequence and length. From the porcine libraries, only a single cDNA clone representing this variant could be isolated, and this lacked several codons at the 3' end as well as the entire untranslated region. The deduced amino acid sequences of the two calponin variants of mouse and pig are aligned and compared with chicken  $\alpha$ -calponin [16] in Fig. 1. According to the terminology previously used for high- ( $h$ -) and low-molecular mass ( $l$ -) calponins observed in human uterus [28] the two mammalian forms will be referred to as  $h_1$ -calponin for the chicken homologue and  $h_2$ -calponin for the new species.

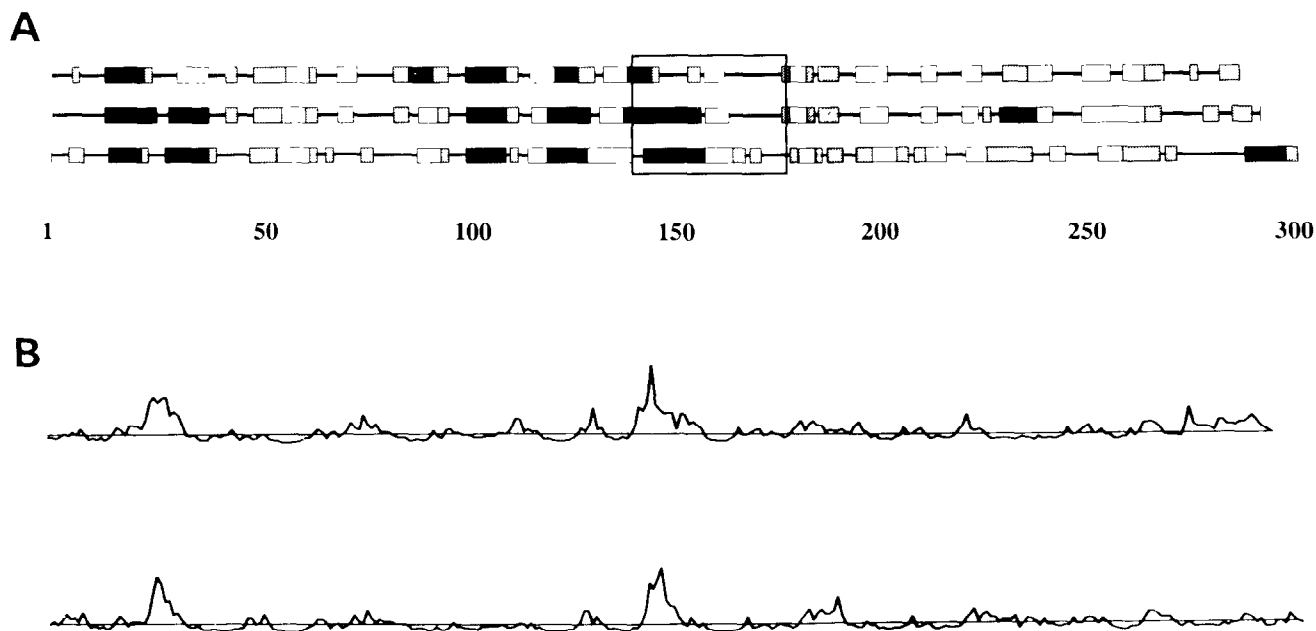


Fig. 2. Structural predictions made from the protein sequences. (A) Secondary structure predictions of chicken- (top),  $h_1$ - (middle) and  $h_2$ -calponin (bottom); solid box:  $\alpha$ -helix, dotted box:  $\beta$ -turn; open box:  $\beta$ -sheet; solid line: random coil. (B) Surface probability plots of  $h_1$ - (top) and  $h_2$ -calponin (bottom). The boxed area in (A) designates the actin-binding region from residue 145–182 in the chicken sequence as proposed by Mezgueldi et al [17]. Numbers indicate approximate positions of amino acid residues.

### 3.2. Structural analyses made from the amino acid sequences

Predictions of secondary structure and surface probability are presented schematically in Fig. 2. Comparison of mammalian  $h_1$ - and  $h_2$ - with chicken calponin revealed several regions of structural similarity, predominantly in the N-terminal half of the molecules (Fig. 2A). In Fig. 2B, surface probability plots of  $h_1$ - and  $h_2$ -calponin are shown. In each of the variants, two strongly hydrophilic domains can be identified which are likely to be exposed on the outer surface of the proteins. Both regions are found at identical positions in chicken calponin [16], and one of them, located approximately between residues 140 and 160, maps to the actin-binding region suggested by Mezgueldi et al. [17].

### 3.3. Expression of mRNA encoding $h_1$ - and $h_2$ -calponin in smooth muscle

The ratio of the expression levels of  $h_1$ - and  $h_2$ -calponin in mouse uterus was estimated from quantitative Northern blot analysis. Two identical blots of mouse uterus mRNA were hybridized separately with N-terminal  $h_1$ - and  $h_2$ -calponin cDNA probes of the same length and specific activity under conditions avoiding cross-hybridization. For each probe a single band could be detected, and their molecular weights corresponded to the sizes of the respective cDNA clones (Fig. 3). Laser densitometry of the blots gave a ratio of  $h_2$ : $h_1$ -calponin mRNAs in the order of 1:10. In addition, we checked for expression of  $h_2$ -calponin mRNA in porcine stomach, aorta and uterus. Using porcine  $h_1$ - and  $h_2$ -calponin-specific probes on Northern blots of RNA from these tissues we obtained essentially the same hybridization pattern as was demonstrated for mouse uterus. However, the relative signal intensity for  $h_2$ -calponin mRNA was significantly lower in the pig samples and could be detected only after prolonged exposure times (data not shown). Under the conditions employed, we found no detectable hybridization of either probe to chicken gizzard mRNA, run in a control lane. Moreover, in no case were we able to detect mRNA of lower molecular weight that could encode the  $l$ -calponin variant [16,28].

### 3.4. Two-dimensional gel electrophoresis of calponin expressed in *E. coli*

To determine the position of  $h_2$ -calponin relative to  $h_1$ -calponin on two-dimensional electrophoresis gels, both mouse calponin cDNAs were expressed in *E. coli* and the protein products were designated CAL-1 (for  $h_1$ -calponin) and CAL-2 (for  $h_2$ -calponin). A polyclonal antibody directed against porcine calponin was used to detect both proteins in immunoblots (Fig. 4). As shown, CAL-2 exhibited a significantly more acidic isoelectric point (pI) and a slightly higher apparent molecular mass than CAL-1. The pI values estimated from these blots were in good agreement with the values predicted from

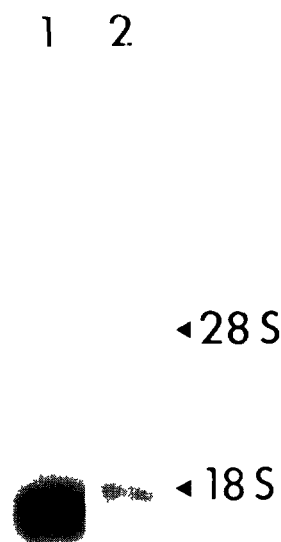


Fig. 3. Expression of  $h_1$ - and  $h_2$ -calponin mRNA in mouse uterus. 2  $\mu$ g of poly(A)<sup>+</sup> RNA were run in each lane of the RNA blot and hybridized with mouse specific probes. Lane 1, hybridized with a 327 bp-fragment representing the 5'-end of the  $h_1$ -calponin cDNA clone; lane 2: hybridized with a 300 bp-fragment representing the 5'-end of the  $h_2$ -calponin cDNA clone. The positions of 18S- and 28S rRNA are indicated to the right. The blot was exposed to X-ray film for 6 h.

the sequences (Table I). In addition, by co-electrophoresis and Western blotting we could demonstrate that the expressed CAL-1 and the native  $h_1$ -calponin isolated from mouse tissue migrated in the same position on two dimensional electrophoresis gels (not shown). No protein reaction with the calponin antibody could however be detected in two-dimensional gels of smooth muscle extracts (from pig aorta, stomach, uterus, bladder; mouse aorta, stomach, uterus) in the position predicted for  $h_2$ -calponin.

Table I  
Molecular mass and pI predictions of calponin variants

	Mol. mass (Da)	pI
Chicken <sup>a</sup>	32,390	9.91
Mouse $h_1$	33,335	9.68
Pig $h_1$	33,203	9.93
Mouse $h_2$	33,104	7.82
Pig $h_2$ <sup>*</sup>	31,981	8.33

<sup>\*</sup>Incomplete sequence (see text).

<sup>a</sup>Sequence according to Takahashi and Nadal-Ginard [16]

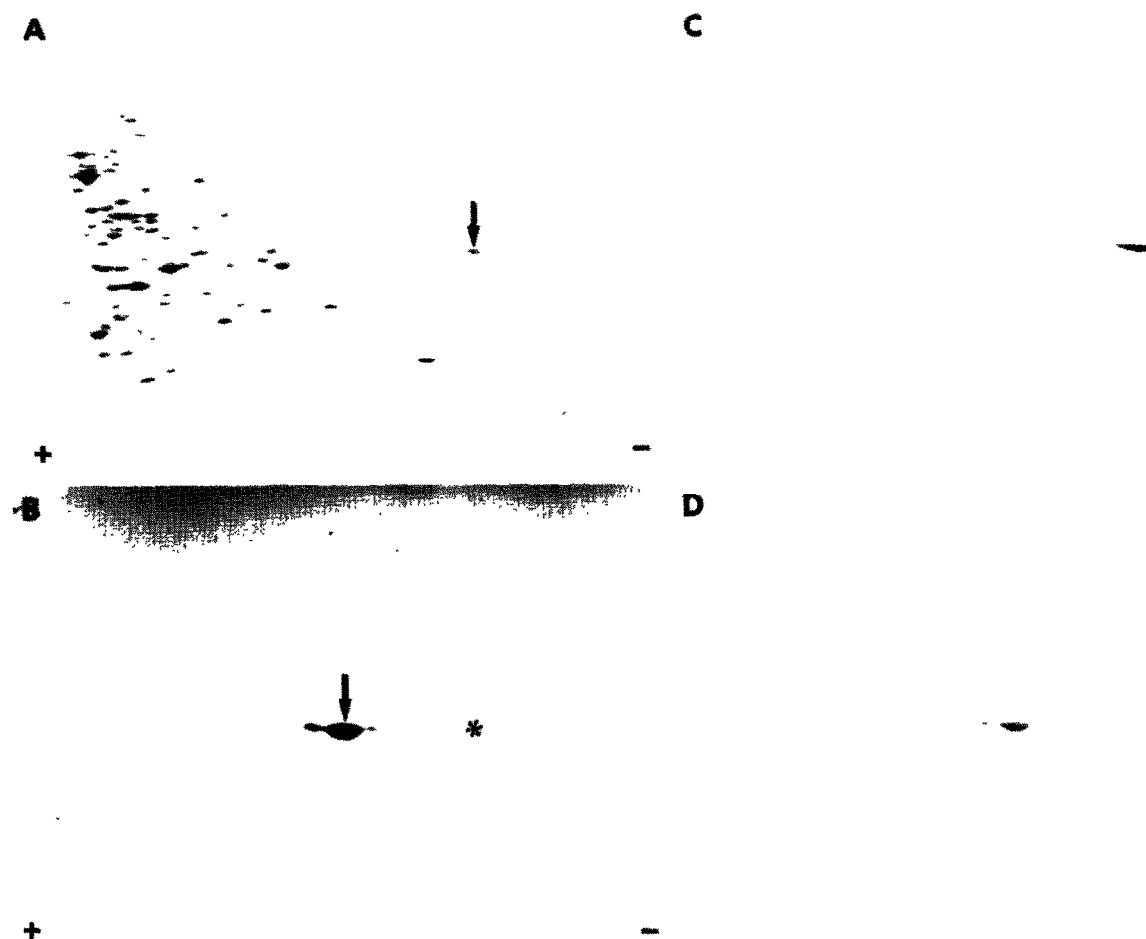


Fig. 4. Two-dimensional gel electrophoresis and immunoblots of calponin variants CAL-1 (A,C) and CAL-2 (B,D) expressed in *E. coli*. (A,B) Silver-stained gels; (C,D) corresponding immunoblots stained with the polyclonal calponin antibody. Note shift in position of CAL-2 (arrow in B) relative to CAL-1 (arrow in A) by about 2 pI units (see Table I). Asterisk in (B) indicates approximate position of CAL-1 and native mouse  $h_1$ -calponin.

#### 4. DISCUSSION

We present here the first data on the primary structures of calponins from mammalian smooth muscle tissues and demonstrate the existence of two genetic variants of the molecule. It has previously been shown [2,14,28] that calponin purified from adult avian and mammalian smooth muscle migrates as multiple isoelectric isoforms on two dimensional electrophoresis gels. These isoforms which are expressed in a developmental-specific fashion arise from a single mRNA species [14] corresponding to  $h_1$ -calponin and acquire their differences in charge via post-translational modifications other than phosphorylation [13,14]. The  $h_2$ -calponin, identified in this study in both pig and mouse, is a distinct variant of calponin with other migration properties. It also shows too many differences in sequence to  $h_1$ -calponin to be explained as a product of alternative splicing of the same mRNA. In the latter sense it

is unrelated to chicken  $\beta$ -calponin of Takahashi and Nadal-Ginard [16] which is smaller than  $\alpha$ -calponin by around 4 kDa due to the exclusion of a 40 amino acid stretch close to the C-terminus of the molecule. In support of the existence of two genes for mammalian  $h_1$ - and  $h_2$ -calponin we obtained two distinct classes of cDNA clones encoding these molecules. Moreover, from a genomic mouse library we also isolated two classes of clones specific for  $h_1$ - and  $h_2$ -calponin, respectively (data not shown).

The different calponins now sequenced (Fig. 1) all show common, repeated motifs in their C-terminal halves (double-ended arrows in Fig. 1) and a hypervariable C-terminus. Interestingly, the hypervariability in mammals applied only to the two calponin variants in the same species, whereas the C-termini of the individual variants in mouse and pig show a high degree of homology (Fig. 1). According to studies with proteolytic fragments of chicken calponin the C-terminal part

of the molecule, embracing amino acids 183–292, that includes the latter half of the first repeat sequence, the following two repeats and the variable C-terminus, is not required for the inhibition of actomyosin MgATPase activity [12,17]. Whether this C-terminal tail piece is of any importance for the function of the molecule has yet to be established.

A noted feature of calponin is its capacity to bind F-actin [1,2]. Mezgueldi et al. [17] recently showed the actin-binding site in chicken calponin to be located at the C-terminal end of the N-terminal 22-kDa fragment which is obtained by limited chymotryptic digestion [4]. At positions 142–147 the authors highlighted a motif VKYAEK (marked with a solid bar in Fig. 1) with homology to the sequence LKHAET that has been found in several actin-binding proteins and is thought to be involved in actin-binding [29]. It is noteworthy that in the two  $h_1$ -calponin sequences (Fig. 1) the VKYAEK motif is conserved but slightly deviates in the  $h_2$ -calponin sequences where it is changed to VKYSEK.

Secondary structural analysis of the protein sequences revealed several regions of similarity to chicken calponin (see Fig. 2A). The C-terminal third carrying the three quasi repeats appears to exhibit a higher variability than the N-terminal two thirds of the calponin molecules. The binding sites for calmodulin, tropomyosin and F-actin are considered to be located in this N-terminal portion [1,2,4,17]. The surface profile (Fig. 2B) correlates one of the two strongly hydrophilic stretches of the molecules (around position 150) with the putative actin-binding area [17]. The role of the other hydrophilic region (around position 30) is as yet unclear. From the data reported by Mezgueldi et al. [17] all three binding activities of calponin are thought to reside in the fragment between residues 61–182. Thus, it seems to be unlikely that the tropomyosin- or calmodulin-binding domain is located around this exposed region at the N-terminus of the molecule. Binding studies using engineered fragments of calponin have to be performed to settle this question.

As demonstrated by Northern blot analysis the  $h_2$ -calponin mRNA represents a low abundant species in all tissues tested. From the ratio of  $h_2$ - to  $h_1$ -calponin-specific signals on the Northern blots, around 1:10 (Fig. 3), traces of the  $h_2$ -calponin protein on Western blots of smooth muscles tissues would be expected. However, we failed to detect the protein with our polyclonal antibody, a result that may indicate a down-regulation of expression of  $h_1$ -calponin at the translational level.

A future aspect of our work will focus on the determination of calponin sequences involved in binding to partner molecules. To pinpoint functional motifs we are currently constructing and expressing deletion mutants of both calponin variants in *E. coli* for analysis in vitro.

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