Molecular cloning, functional expression and tissue distribution of the cDNA encoding frog skeletal muscle calsequestrin

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We have cloned, sequenced and expressed the cDNA encoding frog skeletal muscle calsequestrin. The processed frog calsequestrin is 398 residues long, with an M_r of 45 941 (unglycosylated form), and exhibits 77 % sequence similarity with its rabbit counterpart. Consensus sequences for glycosylation and phosphorylation of the protein were conserved. Compared with rabbit calsequestrin, the mature amphibian protein has peculiar structural properties, which include (i) a higher content of negatively charged residues (142 versus 109), and (ii) a striking repeat sequence at the C-terminal region of 44 aspartic acid residues. Furthermore, this is the first report on the expression of calsequestrin cDNA in COS-1 cells; the expressed protein exhibited an M_r and antigenic properties which were indistinguishable from those of the native protein. In addition, it was capable of binding ^{45}Ca in a ligand overlay. Northern blot analysis of frog skeletal muscle, liver, heart and brain RNA showed that the protein is mainly expressed in skeletal muscle. The high density of negative charges at the C-terminus might constitute high-capacity low-affinity Ca^{2+} -binding sites, which may account for the higher Ca^{2+} -binding capacity of frog calsequestrin compared with other members of the calsequestrin family (56 mol/mol versus 40–44 mol/mol of protein).

INTRODUCTION

Skeletal muscle sarcoplasmic reticulum (SR) is an intracellular membrane compartment which controls the myoplasmic Ca^{2+} concentration, thereby playing a crucial role in the excitation–contraction mechanism (Endo, 1977). Morphologically, SR consists of two portions: (i) free SR, made up of longitudinal tubules and the non-junctional membrane of the terminal cisternae, and (ii) junctional SR, comprising the region of terminal cisternae associated with the transverse tubules via feet structures (Franzini-Armstrong, 1980). Muscle relaxation is due to ATP-dependent Ca^{2+} uptake into the SR (Inesi, 1985), and release of Ca^{2+} bound to calsequestrin via a Ca^{2+} channel initiates contraction (Endo, 1977; Somlyo *et al.*, 1985). Calsequestrin is a moderate-affinity, high-capacity Ca^{2+} -binding protein (MacLennan & Wong, 1971) which resides in the matrix of terminal cisternae, closely associated with (anchored to) the intraluminal face of the junctional SR (Saito *et al.*, 1984; Kawamoto *et al.*, 1986; Franzini-Armstrong *et al.*, 1987; Mitchell *et al.*, 1988; Damiani & Margreth, 1990). Calsequestrin seems to act as an intraluminal Ca^{2+} buffer, and may also facilitate the transfer of Ca^{2+} through the junctional membrane during the coupling of excitation to Ca^{2+} release (Ikemoto *et al.*, 1989; Volpe & Simon, 1991). Despite the wealth of physicochemical, biochemical and functional studies that have been carried out on calsequestrins, the exact mechanism and protein domain(s) involved in Ca^{2+} binding remain elusive (Ikemoto *et al.*, 1974; Ohnishi & Reithmeier, 1987; Fliegel *et al.*, 1987; Scott *et al.*, 1988).

While the biochemical and functional characterization of the protein components of the SR has been mainly performed on microsomal preparations from mammalian muscles, our information on muscle physiology derives to a large extent from experiments carried out on frog muscle fibres, and general conclusions have been drawn without taking into account species-specific differences. As far as the morphological and biochemical properties of terminal cisternae are concerned, there are dif-

ferences between amphibians and mammals (Franzini-Armstrong, 1977; Volpe *et al.*, 1988). In particular, it has been shown that calsequestrin and a set of high- M_r feet proteins (ryanodine receptor/ Ca^{2+} channel) from frog skeletal muscle SR possess unique structural and functional properties (Damiani *et al.*, 1986; Volpe *et al.*, 1988; Airey *et al.*, 1990; Lai & Meissner, 1991). Frog calsequestrin cross-reacts only weakly with anti-(rabbit calsequestrin) antibodies (Damiani *et al.*, 1986), exhibits a more pronounced shift in the apparent M_r depending on the pH of the SDS-containing separating gel (Damiani *et al.*, 1986) and displays a higher Ca^{2+} -binding capacity than its rabbit counterpart (56 mol of Ca^{2+} /mol versus 40–44 mol of Ca^{2+} /mol of protein respectively) under ionic conditions that mimic those present in the terminal cisternae at rest (Volpe *et al.*, 1988; Volpe & Simon, 1991).

In an attempt to elucidate the structural basis for the difference in the Ca^{2+} -binding properties of mammalian and amphibian calsequestrin, we determined the primary structure of frog (*Rana esculenta*) skeletal muscle calsequestrin. Comparison of the deduced amino acid sequence of frog fast-twitch skeletal muscle calsequestrin with that of rabbit skeletal muscle revealed that the two proteins are overall 77 % similar. The most interesting structural feature of frog calsequestrin consists of a C-terminal repeat of 44 aspartic acid residues which might account for the high Ca^{2+} -binding capacity of this calsequestrin.

EXPERIMENTAL

Materials

cDNA synthesis, mRNA purification and T₇ sequencing kits, *Eco*RI adaptors, DNA standards, mung bean nuclease, agarose, dextran sulphate, DEAE-dextran and λ gt11 cloning vector were from Pharmacia–LKB Biotechnology; restriction endonucleases, T₄ DNA ligase, exonuclease III, and the Klenow fragment of DNA polymerase I were from Boehringer–Mannheim; nitrocellulose and nylon membranes were from Schleicher and Schuell

Abbreviation used: SR, sarcoplasmic reticulum.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X64324.

and Amersham; [α - 32 P]dATP was from Amersham; Bluescript, λ ZAP II cloning vector and the Gigapack Gold packaging extracts were from Stratagene; *N*-lauroylsarcosine, guanidine isothiocyanate, diethyl pyrocarbonate and formaldehyde were from Sigma; Dulbecco's modified Eagle's medium and fetal bovine serum were from Gibco Laboratories; tissue culture supplies were from Falcon and Costar; acrylamide and *NN'*-bisacrylamide were from Merck; alkaline phosphatase-conjugated anti-(guinea pig IgG) antibodies were from Sigma and Zymed Inc.; all other chemicals were reagent grade.

RNA and DNA manipulations

Total RNA from frog tight leg was isolated by the guanidinium isothiocyanate procedure as described by Maniatis *et al.* (1989), and poly(A)⁺ RNA was purified by oligo(dT) spun column chromatography using the kit supplied by Pharmacia. Total RNA was separated by agarose-formaldehyde gel electrophoresis and electrophoretically transferred on to a nylon membrane. Hybridization to 32 P-labelled cDNA probes was carried out as described by Whal *et al.* (1979). $20 \times$ SSCP was prepared, which contained 2.4 M-NaCl, 2.9 M-sodium citrate, 3.8 M-KH₂PO₄ and 10 mM-EDTA, pH 7.2. Radiolabelling of the cDNA probes was carried out by the random primer method (Feinberg & Vogelstein, 1983). Templates for sequencing were prepared in the Bluescript vector. The exonuclease III/mung bean nucleases deletion mutants were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using the T₇ polymerase sequencing kit supplied by Pharmacia. All other manipulations of nucleic acids were carried out according to standard protocols (Maniatis *et al.*, 1989).

Isolation of frog cDNA clones

The cDNA libraries were constructed from 10 μ g of poly(A)⁺ RNA isolated from frog fast-twitch tight leg muscles. cDNA was synthesized with the cDNA synthesis kit supplied by Pharmacia, ligated to *NotI/EcoRI* adaptors, and cloned into λ gt11 and λ ZAPII vectors, and packaging *in vitro* of 3–4 litre of ligation mixture was performed using Gigapack Gold packaging extracts. The cDNA library in λ gt11 yielded 2×10^6 independent clones, which were screened using affinity-purified anti-(frog calsequestrin) antibodies (Treves *et al.*, 1991) according to the procedures described by Young & Davis (1983). Restriction endonuclease fragments from immunopositive clones were used as probes to screen size-selected cDNA libraries in λ ZAPII to isolate the full-length clone (Maniatis *et al.*, 1989).

Expression of the full-length cDNA in COS-1 cells

The full-length frog calsequestrin cDNA carrying the *NotI/EcoRI* adaptors was cloned into the *EcoRI* site of the expression vector pMT2 (Kaufman *et al.*, 1989), in either the correct or the reverse orientation. The plasmid DNA was transfected into COS-1 cells by the DEAE-dextran procedure, as described by Kaufman *et al.* (1989) and detailed by Vilsen *et al.* (1989). The subcellular fractions were obtained by differential centrifugation 48–72 h after transfection of the cells (Maruyama & MacLennan, 1988; Vilsen *et al.*, 1989). The microsomal proteins as well as the total cell homogenate were subjected to slab gel SDS/PAGE according to Laemmli (1970) and electrophoretically transferred to nitrocellulose paper. Indirect immunoenzymic staining of the Western blot was carried out as described by Vilsen *et al.* (1989).

45 Ca ligand overlay

45 Ca ligand overlay was carried out as described by Maruyama *et al.* (1984). The nitrocellulose membrane was washed at room temperature for 30 min in a medium containing 5 mM-imidazole,

pH 6.8, 60 mM-KCl and 5 mM-MgSO₄, and then incubated in the same buffer plus 1 μ Ci of 45 CaCl₂/ml for 20 min. The membrane was rinsed with 100 ml of distilled water followed by 100 ml of 50% ethanol, dried and exposed to X-Omat film for 5–6 days.

RESULTS

cDNA isolation and sequencing

Screening of the cDNA expression library in λ gt11 carried out with an affinity-purified anti-(frog calsequestrin) antibody led to the isolation of five clones. The 205 bp fragment defined by nucleotides 210–415 (Fig. 1*b*) isolated from the 1.5 kb FCS1 clone was subcloned into Bluescript and sequenced to determine sequence similarity with rabbit calsequestrin cDNA. This fragment encodes an amino acid sequence highly similar to that of rabbit calsequestrin (Fliegel *et al.*, 1987). Thus this cDNA was used as a probe to isolate the full-length clone (2.45 kb) from a cDNA library in λ ZAPII. The full-length calsequestrin clone was sequenced in both directions by the exonuclease III/mung bean nuclease deletion method. Figs. 1*a*) and 1*b*) show the restriction map and sequencing strategy for the frog calsequestrin clone and the nucleotide and deduced amino acid sequences respectively. There is one open reading frame of 1263 nucleotides, bounded by 246 bp at the 5' untranslated region and 943 nt at the 3' untranslated region. The 5' untranslated region is rich in G+C residues; this is a characteristic of similar regions of other SR proteins (MacLennan *et al.*, 1985). The initiator ATG at position +1 was found within a sequence (GAGAGGATGA) which has features resembling the consensus initiator sequence CCA(A/G)-CCATGG (Kozak, 1984). There is, however, an inframe stop codon at position -33 and no other ATG codon before methionine at position 1; thus this ATG ought to constitute the initiator codon. Two polyadenylation signals (Proudfoot & Brownlee, 1976) were found 9 and 546 nt away from the TAA stop codon.

Amino acid sequence analysis

The deduced amino acid sequence of unprocessed frog skeletal calsequestrin begins with a 22-residue-long sequence which has properties common to signal sequences. It contains hydrophobic residues (Fig. 1*b*) bound by positively charged residues, with a small amino acid (Gly or Ala) at the end, which usually precedes the cleavage site of many signal sequences (Von Haigje, 1985). The processed frog calsequestrin is made up of 398 residues with a predicted *M_r* (unglycosylated form) of 45941, a value much smaller than that of 72000 observed in a Laemmli gel system (see Figs. 3*a*, and 3*b*, lanes 1 and 3). Within the first 35 residues the deduced *N*-terminal sequence of the processed protein matches that determined from the native protein, except that at position 30 a tyrosine rather than a phenylalanine is present (Treves *et al.*, 1991). The deduced amino acid sequence of frog skeletal muscle calsequestrin is typical of other calsequestrins, but also shows important differences. It contains a high percentage of acidic residues, 50 glutamates and 92 aspartates, which are mainly clustered to form duplets (15), triplets (4), quadruplets (2) and a quintuplet (1). It also has a unique repeat of 44 aspartic acid residues at the *C*-terminus. These results are unlikely to be artifacts, since Table 1 shows that the content of the aspartate residues of deduced sequence is, within experimental error, similar to that determined from the native protein. Overall the acidic residues are located in the last two-thirds of the sequence. Five tryptophan residues are also located at the *C*-terminal region. Basic residues are dispersed at the *N*-terminal part of the molecule, with the occurrences of two lysine doublets, and two arginine and lysine doublets.

Fig. 1. Characterization of cDNA encoding frog skeletal muscle calsequestrin

(a) Restriction map and sequencing strategy of the 2.4 kb cDNA clone of frog skeletal muscle calsequestrin. The open box indicates the coding region; the hatched box indicates the signal sequence. (b) Nucleotide and deduced amino acid sequences of frog skeletal calsequestrin. The nucleotides are numbered positively from the first residue of the initiator methionine. The nucleotides of the 5' non-translated region are numbered negatively in the 3' to 5' orientation. The N-terminal sequence determined from the purified protein (Treves *et al.*, 1991) is underlined; potential phosphorylation and glycosylation sites are boxed; ▲▲▲, polyadenylation signal sequence.

A search for consensus sequences compatible with post-translational modification of the protein was positive for potential glycosylation [Asn-Xaa-Thr(Ser)] and cyclic AMP-dependent protein kinase phosphorylation sites (Feramisco *et al.*, 1980) at positions 340 and 251 (Fig. 1b) respectively. Secondary structure predictions of the protein are similar to that of other

Table 1. Amino acid composition of frog skeletal muscle calsequestrin: comparison between native protein and deduced amino acid sequence

Amino acid	Composition (mol %)	
	Native protein*	Protein deduced from cDNA
Ala	5.30	5.5
Cys	2.98	0
Asp	19.75	23.1
Glu	12.52	12.6
Phe	4.01	4.8
Gly	14.78	3.8
His	2.40	2.5
Ile	7.46	6.0
Lys	5.78	5.3
Leu	7.46	8.8
Met	0.24	1.3
Asn	—	2.5
Pro	3.17	4.8
Gln	—	1.3
Arg	1.44	1.5
Ser	3.16	3.3
Thr	2.97	2.8
Val	6.01	5.3
Trp	—	1.3
Tyr	3.13	3.8

* Damiani *et al.* (1986).

EDGLDFPEYD	GEDRVIHSL	KNYKAALKKY	EVLALLYHEP	IGDDKASQKQ	FEMEELILEL	60
AAQVLEDKGV	GFGLVDEDD	AAVAKKLGLD	EEDSIYVFKD	DEMIEYDGEF	SADTLVEFLL	120
DVLEDPVEFI	DGSHELAAFE	NLDDEPKLIG	YFKNEDSEHY	KAYEDAAEEF	HPYIPFFATF	180
DAKVAKTLTL	KLNEIDYVEP	FHDEPITIPS	KPNSEKEIVD	FLHQHQRPTL	RKLRPDSHYE	240
TWEDDLNGIH	IVAFAEEDDP	DGYEFLQIK	EVAEDNTDNP	DLSIIWIDPE	DFPLLIPIWE	300
EKFIDLSRP	HIGVNVTD	DSVWMDDE	EDLPTVDELE	DWIEDVLEGE	VNTEDDDDD	360
DDDDDDDDDD	DDDDDDDDDD	DDDDDDDDDD	DDDDDDDDDD	DDDDDDDDDD	DDDDDDDDDD	398

Fig. 2. Predicted secondary structure of frog skeletal muscle calsequestrin

Analysis of the deduced amino acid sequence of frog skeletal muscle calsequestrin was carried out using the GENEPRO computer program. Secondary structural predictions were carried out with the Chou & Fasman (1978) program. Potential α -helix (====), β -sheets (----) and β -turns (···) were predicted using a window of six, five and four residues respectively (Chou & Fasman, 1978).

sarco(endo)plasmic Ca^{2+} storage proteins (calsequestrin and calreticulin). The hydropathy profile (Kyte & Doolittle, 1982) with a window of 19 amino acids does not display any putative transmembrane segments (results not shown). Computer-assisted analysis of the deduced frog calsequestrin sequence with the Chou & Fasman (1978) program predicts the presence of α -helix and β -sheets throughout the protein, except at the C-terminus, which shows strong potential to form β -turns (Fig. 2).

Expression of the cDNA

To confirm the identity of the calsequestrin clone, we expressed the cDNA in COS-1 cells. As indicated by the Western blot, this cell line apparently does not contain endogenous calsequestrin, and was therefore considered to be an appropriate expression system for the frog calsequestrin clone. Fig. 3 shows a Western blot of subcellular fractions from COS-1 cells transfected with the full-length cDNA clone inserted in the pMT2 vector either in the correct (Figs. 3a and 3b, lane 1; Fig. 3c, lanes 1 and 2) or in the reverse orientation (Figs. 3a and 3b, lanes 2). ^{45}Ca ligand overlay of the total cell homogenate revealed that the expressed

Fig. 3. Expression of the cDNA encoding frog skeletal muscle calsequestrin in COS-1 cells

The total homogenate and the microsomal and cytosolic fractions were run on an SDS/7% polyacrylamide gel prepared according to Laemmli (1970), and then transferred on to nitrocellulose paper. (a) Autoradiogram of ^{45}Ca overlay and (b) Western blot with an anti-(frog calsequestrin) antibody of the total homogenate from COS-1 cells transfected with the cDNA inserted in the expression vector in either the correct (lanes 1) or the reverse (lanes 2) orientation. Lanes 3 (a and b) contain calsequestrin purified from skeletal muscle. (c) Western blot with anti-(frog calsequestrin) antibody of the cytosolic (lane 1) and microsomal (lane 2) fractions from COS-1 cells transfected with the cDNA inserted in the correct orientation in the expression vector.

calsequestrin binds Ca^{2+} (Fig. 3a, lane 1) and has an apparent M_r similar, if not identical, to that of the native protein (Fig. 3a, lane 3). The band which bound ^{45}Ca was also reactive with affinity-purified anti-(frog calsequestrin) antibodies (Fig. 3b, lane 1). COS-1 cells transfected with the expression vector containing the cDNA in the reverse orientation failed to express the protein (Figs. 3a and 3b, lanes 2).

Fig. 4. Northern blot analysis of RNA from frog skeletal muscle, liver, brain and heart

Total RNA (20 μg) was electrophoretically separated in a formaldehyde-agarose gel and transferred on to a nylon membrane. Ribosomal bands corresponding to 4.7 and 1.9 kb are indicated. Hybridization was carried out as described in the Experimental section using the full-length cDNA of frog calsequestrin. Lane A, skeletal muscle; B, liver; C, heart; D, brain.

Fig. 5. Southern blot of frog genomic DNA

DNA (40 μg) was digested with *Hind*III (lane A, *Sac*I (lane B) and *Bam*HI (lane C), separated on an agarose gel, and transferred on to a nylon membrane as described in the Experimental section.

The expressed frog calsequestrin was present both in the microsomal fraction and in the high-speed supernatant (Fig. 3c, lanes 1 and 2 respectively). It was not, however, secreted into the cell culture medium (results not shown). In a parallel set of experiments in which COS-1 cells were transfected with the cDNA encoding skeletal muscle Ca^{2+} -ATPase, we observed that, in contrast to calsequestrin, the Ca^{2+} -ATPase was recovered only in the microsomal fraction.

Northern blot and tissue distribution

Northern blot analysis was carried out to examine whether calsequestrin is expressed in different frog tissues. Portions of 20 μg of total RNA isolated from muscle, liver, brain and heart were probed with the full-length cDNA encoding calsequestrin. At high-stringency washes (65 $^{\circ}\text{C}$, $0.2 \times \text{SSCP}$) the calsequestrin probe bound to a 2.5 kb transcript from skeletal muscle (Fig. 4, lane A), while no clear bands were present in 20 μg of total RNA isolated from liver, heart and brain. Washing the membranes at low stringency (42 $^{\circ}\text{C}$, $2 \times \text{SSCP}$) a higher hybridizing band was

detected in skeletal muscle RNA, while in the other tissues no hybridizing bands were observed (results not shown).

Southern blot analysis of genomic DNA

The mammalian genome contains several calsequestrin-like sequences (Scott *et al.*, 1988), and thus we examined whether frog genomic DNA contains sequences identical to the calsequestrin gene. Genomic DNA was digested with restriction enzymes, transferred to solid support, and probed with the ³²P-labelled full-length calsequestrin cDNA. The autoradiograph shows two or three hybridizing bands in each digest, indicating the existence of calsequestrin-like genomic sequences (Fig. 5).

DISCUSSION

Calsequestrins are high-capacity Ca²⁺-binding proteins involved in calcium homeostasis in excitable tissues (MacLennan & Wong, 1971; Mitchell *et al.*, 1988; Volpe *et al.*, 1990). In this study we have cloned, sequenced and expressed the cDNA encoding frog skeletal muscle calsequestrin. Overall frog calsequestrin shows 77% sequence similarity to its rabbit counterpart, and could be aligned with the rabbit protein by introducing only one gap (Fig. 6). Mature frog calsequestrin exhibits 70 substitutions, of which 47 are non-conservative and 23 are conservative. We also found that the number and localization of positively charged residues of the two calsequestrins are similar. The substitutions are distributed throughout the molecule, and most likely do not drastically modify the conformation of the protein, since they mainly occur in non-structural regions. Rabbit calsequestrin contains two potential phosphorylation sites, but only that defined by residues 226–229 is conserved in the frog protein (Fig. 6). Although the physiological role of calsequestrin phosphorylation, if any, is not known, the conservation of this potential phosphorylation site over millions of years of evolution suggests that it may be relevant to protein function.

The most interesting feature of the frog calsequestrin sequence concerns both the content and distribution of acidic residues. Here we show that the content of acidic residues of the deduced amino acid sequence is similar to that of native frog calsequestrin (Table 1), and is 37% higher than that of the rabbit calsequestrin

(142 versus 103). The localization of the acidic residues over the molecule is virtually identical in both adult calsequestrins, except that an extra tail of 39 aspartate residues is present at the C-terminus of frog calsequestrin. We also report, for the first time, the expression of frog calsequestrin cDNA in COS-1 cells, a non-muscle cell line. The expressed protein was immunologically and functionally indistinguishable from muscle calsequestrin, and migrated with an apparent *M_r* identical to that of the native protein. Altogether, these results seem to exclude the possibility that the C-terminal aspartate repeat is the product of a cloning artifact. Such a unique repeat of aspartate residues may be responsible for the higher Ca²⁺-binding capacity of frog calsequestrin compared with that of other sarco(endo)plasmic Ca²⁺ storage proteins (MacLennan & Wong, 1971; Ostwald & MacLennan, 1974; Fliegel *et al.*, 1987; 1989; Mitchell *et al.*, 1988; Scott *et al.*, 1988; Smith & Koch, 1989; Treves *et al.*, 1990). At variance with this possibility, however, is the report of a 33-aspartate-long repeat at the C-terminus of chicken skeletal muscle calsequestrin/aspartactin (Clegg *et al.*, 1988; Yazaki *et al.*, 1990), a protein with a maximal Ca²⁺-binding capacity of 20 mol Ca²⁺/mol (Damiani *et al.*, 1990). However, there are factors which may explain such an apparent discrepancy. First of all, one should consider that the aspartate repeat of calsequestrin/aspartactin is 35% shorter than that occurring in frog calsequestrin. Another possibility, although highly speculative, is that the aspartate repeat of chicken calsequestrin is cleaved off during biosynthesis. Finally, one cannot exclude the possibility that, as for dog cardiac calsequestrin (Mitchell *et al.*, 1988), the maximal Ca²⁺-binding capacity of chicken skeletal muscle calsequestrin has been underestimated.

Can functions other than Ca²⁺-binding be attributed to the aspartate repeat of frog calsequestrin? Such a high density of negative charges may be responsible for the interaction of calsequestrin with either components of the Ca²⁺ release mechanism or other cations (Mg²⁺ and protons). During tetanus, approx. 45% of the released Ca²⁺ dissociates from calsequestrin (Somlyo *et al.*, 1985; Volpe & Simon, 1991), and simultaneously the Mg²⁺ content of the terminal cisternae increases (Somlyo *et al.*, 1985). The driving force behind the Mg²⁺ influx is not known, but one might speculate that it is generated by the high density of negative charges of the aspartate repeat localized just at the inner leaflet of the junctional membrane. Further studies on calsequestrin deletion mutants lacking the C-terminus may clarify the role of this domain in calsequestrin function.

It is noteworthy that the frog calsequestrin expressed by COS-1 cells was recovered not only in the microsomal pellet but also in the high-speed supernatant, a fraction comprising the cytoplasm. In skeletal muscle, calsequestrin appears to be anchored to the SR (Saito *et al.*, 1984; Kawamoto *et al.*, 1986; Franzini-Armstrong *et al.*, 1987; Damiani & Margreth, 1990) via protein components of the junctional SR. Although we do not exactly know why calsequestrin is present in the high-speed supernatant, it might be suggested that anchoring devices responsible for maintaining calsequestrin within terminal cisternae during preparative procedures are not expressed in COS-1 cells. This concurs with the lack of a typical endoplasmic reticulum retention (anchoring?) signal (Lys-Asp-Glu-Leu or similar) in the calsequestrin sequence.

Our Northern blot analysis shows that the protein encoded by the cloned cDNA is expressed mainly in frog skeletal muscle, and is not expressed in non-muscle tissues or in heart. As far as the calsequestrin-like sequences present in the frog genome are concerned, we do not know whether they represent functional genes or pseudo-genes. In a recent report (Tidball, 1990) the purification of a frog 80000-*M_r* glycoprotein, named myonexin, has been described. This protein was suggested to act as a

Fig. 6. Comparison of the deduced amino acid sequences of frog and rabbit skeletal muscle calsequestrin

Comparison is with the neonatal form of rabbit skeletal muscle calsequestrin (Fliegel *et al.*, 1987). Mature rabbit calsequestrin is identical to the neonatal form, except that at the C-terminus it lacks seven aspartic acid residues and ends with a D/E reversal. Identical residues and conservative substitutions are indicated by colons and by points respectively. Signal sequences are numbered negatively.

fibronectin receptor at the myotendineous junctions. Within the first 20 *N*-terminal residues, myonexin displays 90 % identity with frog skeletal calsequestrin (the present study). Nevertheless, myonexin cannot be classified as a true calsequestrin, as it does not exhibit Ca^{2+} -dependent binding to phenyl-Sepharose (Tidball, 1990), the latter being an invariant property shared by all members of the calsequestrin family examined so far (Damiani *et al.* 1990). Further studies are required to determine the genetic and functional relationship between these two proteins.

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