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Identification of calreticulin isoforms in the central nervous system

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In the present paper we report the cloning and sequencing of the cDNA encoding two calreticulin isoforms from *Xenopus laevis* central nervous system. The two isoforms display 93% identity at the amino acid level. The predicted amino acid sequences of the amphibian calreticulins are very similar (76%) to those of mammalian liver and skeletal muscle. *Xenopus laevis* calreticulins are characterized by a very acidic *c*-terminal domain endowed with the endoplasmic-reticulum retention signal KDEL. The cDNAs of both clones encode an *N*-glycosylation consensus sequence. A third clone of calreticulin was also identified. The restriction map of this clone was clearly distinct from that of the two sequenced clones. These results indicate the existence of multiple calreticulin isoforms in the central nervous system and open questions about their functional role in different cells and/or subcellular compartments.

INTRODUCTION

Calreticulin is a high-capacity Ca^{2+}-binding protein present in the microsomal fraction of most tissues, including heart, skeletal muscle, liver, kidney and brain [1–5]. Because of its abundance, widespread distribution among different cell types and Ca^{2+}-binding characteristics ($B_{max.}$ 40 mol of Ca^{2+}/mol of protein; $k_d \simeq 1$ mM), this protein has been suggested to play a major role in intravesicular Ca^{2+} buffering in non-muscle cells [2].

Calreticulin has been recently cloned and sequenced from mouse liver by Smith & Koch [3] and from rabbit skeletal muscle by Fliegel *et al.* [5]. The mammalian proteins display 96% sequence similarity at the amino acid level; the minor differences are most likely due to species-specific differences. Some evidence exists, however, indicating that a family of calreticulin isoforms may be expressed in mammalian tissues. For example, the purification of calreticulin from rabbit liver consistently demonstrated the presence of a major 59 kDa band as well as the presence of a minor 61 kDa component. Amino acid sequencing of the purified proteins indicated that the major and minor bands had similar, but not identical, *N*-terminal sequences [2]. The expression of different Ca^{2+}-buffering proteins may be of paramount importance for the identification of functionally distinct Ca^{2+} stores.

In the present paper we report the cloning and sequencing of the cDNA encoding amphibian brain calreticulin. We demonstrate that at least two isoforms of the protein are expressed in the central nervous system of *Xenopus laevis*.

METHODS

Isolation of *Xenopus laevis* cDNA clones

A cDNA *Xenopus laevis* brain library in λgt11 was screened with affinity-purified anti-(rabbit liver calreticulin) antibodies [2] as described by Young & Davis [6].

DNA manipulations

Hybridization to ^{32}P-labelled cDNA probes was carried out as previously described [7]; radiolabelling was carried out by the random-primer method using either [α-^{32}P]dATP or [α-^{32}P]dCTP [8]. Templates for sequencing were prepared in the Bluescript cloning vector. The exonuclease III/mung-bean-nuclease deletion mutants were sequenced by the dideoxy chain-termination method [9] using the T7 polymerase sequencing kit supplied by Pharmacia. All other manipulations of nucleic acids were carried out according to standard procedures [10].

Sequence analysis

DNA and amino acid sequences were analysed with the GENEPRO sequence-analysis computer program.

RESULTS

Screening of approx. 3×10^6 plaque-forming units of the *Xenopus laevis* cDNA brain library with affinity-purified anti-(rabbit liver calreticulin) antibodies led to the isolation of a 1.8 kbp clone. The putative clone was digested with *Eco*R1 and yielded two fragments: a 1.3 kbp and a 500 bp fragment. The latter was subcloned into Bluescript and sequenced to determine similarity to calreticulin. Since the cDNA fragment encoded an amino acid sequence highly similar to that of mammalian calreticulin [3,5], it was used to further screen the λgt11 *Xenopus laevis* brain library. After screening approx. 0.5×10^6 plaque-forming units, three putative clones were identified; clone 6, 3 and 8, of approx. 2.6, 2.1 and 2.4 kbp. In Fig. 1 the restriction maps of these clones are compared. Clone 6 had a restriction map which differed from that of the other clones, even though partial sequencing revealed nucleotide similarity, suggesting its cDNA also encoded calreticulin. This clone was not investigated further.

Calreticulin cDNA clones 3 and 8 were sequenced in both directions by the exonuclease III/mung-bean-nuclease deletion method. Clone 3 had one open reading frame of 1233 bp, bound by an 800 bp 3′ untranslated region. It coded for a protein having a molecular mass of approx. 48 kDa. The deduced amino acid sequence begins with a 12-residue-long region which exhibits structural properties common to other signal sequences. It contains hydrophobic residues followed by a small amino acid (alanine) which precedes the cleavage site [11] (Fig. 2). The *N*-terminus of processed rabbit liver calreticulin could be aligned with that of *Xenopus laevis* brain calreticulin beginning with residue +1. Clone 8 was apparently incomplete and lacked the first 11 *N*-terminal amino acids found in the mature protein; it had one open reading frame of 1152 bp bound by a 1200 bp-long 3′ untranslated region. Overall calreticulin clone 3 and 8 are 93% and 89% identical at the amino acid and nucleotide level respectively. The amino acid differences are scattered throughout

The nucleotide sequence data reported will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases.

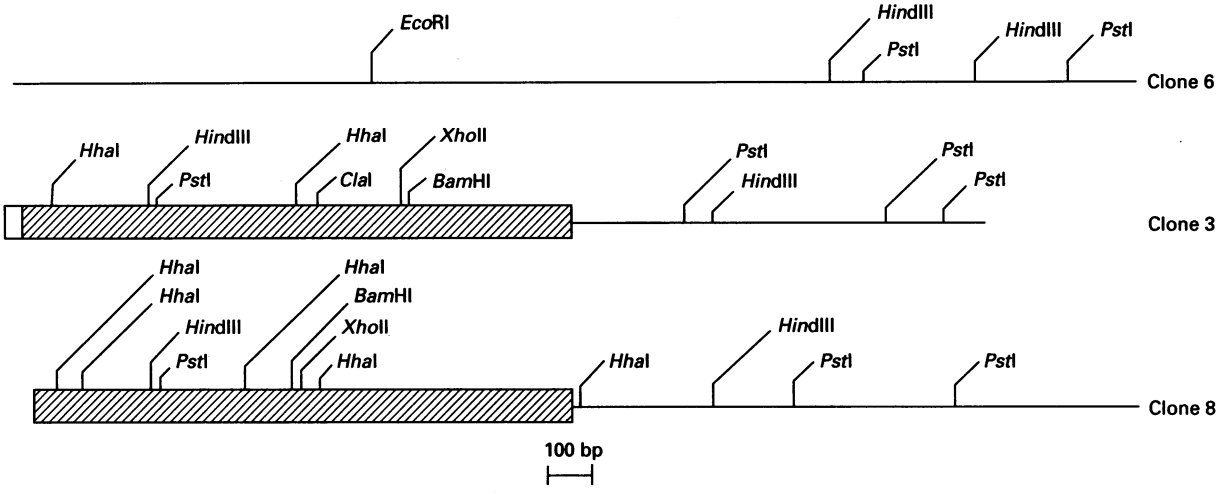

Fig. 1. Restriction endonuclease mapping of calreticulin cDNA clones 6, 3 and 8

The boxed area indicates coding region. The open box indicates the signal sequence. The straight line indicates the non-coding region. Clone 6 was only partially sequenced, subjected to restriction-enzyme digestion and its size determined by gel electrophoresis.

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CR3                                                    LVLPLLAGLCIA   -1

CR3                EPAVYFKEEFTDGDGWTQRWVESKHKTDYGKFKLSAGKFYGDSEKDKG   48
                                 :::::::::::::::.:::::::::::::::::::::
CR8                              DGDGWTQRWVESKHKSDYGKFKLSAGKFYGDSEKDKG   37

CR3   LQTSQDARFYAMSSRFDSFSNKDQTLVVQFSVKHEQNIDCGGGYVKLFPAALEQTEMHEE   108
      ::::::::::::::::::.:::::::::.:::::::::::::::::::::: ::::::: :
CR8   LQTSQDARFYAMSSRFESFSNKDQTLVIQFSVKHEQNIDCGGGYVKLFPADLEQTEMHGE   97

CR3   SEYNIMFGPDICGPPTKKVHVIFQYKKKNLQINKDIRCKDDSFTHLYTLIVRPDNTYEVK   168
      :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CR8   SEYNIMFGPDICGPPTKKVHVIFQYKKKNLQINKDIRCKDDSFTHLYTLIVRPDNTYEVK   157

CR3   IDNSKVESGSLEDDWDFLPPKKIKDPEAKKPDEWDERPKIDDPEDKKPEDWEKPEHIPDP   228
      ::::::::::::.:::::::::::::::::::.:::::::::::::::::::::::::::::
CR8   IDNSKVESGSLEEDWDFLPPKKIKDPEAKKPDDWDERPKIDDPEDKKPEDWEKPEHIPDP   217

CR3   DAVKPEDWDEEMDGEWEPPVIQNPEYKGEWKPRQIDNPDYKGKWIHPEIDNPEYTPDDTL   288
      :::::::::::::::::::::::.  :::::::::::::::::::: :::::::: ::
CR8   DAVKPEDWDEEMDGEWEPPVIQNPDLQGEWKPRQIDNPDYKGKWIHPEMDNPEYTPDSTL   277

CR3   YSYDSFGVLGLDLWQVKSGTIFDNFLMTNDEKHAEEYGNETWGVTKEAEKKMKEQQDEED   348
      :::.::::.:::::::::::::::::::::::: ::::::::::::::::::::::::::::
CR8   YSYESFGVIGLDLWQVKSGTIFDNFLMTNDEKYAEEYGNETWGVTKEAEKKMKEQQDEED   337

CR3   RKKQEEEEKTRKEEEPQEEEDEDDDDEEEKEEEKEEEEEEDEEETPLKDEL           399
      :::::.::   :::::::::::   :::::::::::::::::::: :: ::::
CR8   RKKQEDEENKQKEEEPQEEED---DDEEEKEEEKEEEEEEDEE-TPQKDEL           384
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Fig. 2. Comparison of the deduced amino acid sequences of calreticulin cDNA clones 3 and 8

Identical residues and conservative substitutions are indicated by colons and full stops respectively. The signal sequence is negatively numbered. The potential glycosylation site is boxed.

the molecule. Altogether, there are nine conservative and 15 non-conservative substitutions. In particular, the number of positively charged residues is similar (44), except that clone 8 has a lysine-for-glutamine substitution at position 244. There are few deletions, which are restricted to the *C*-terminal region and mainly involve negatively charged residues: starting from amino acid 12, clone 3 contains a total of 111 negatively charged residues as opposed to the 106 present in clone 8. Clone 3 has an extra glutamate residue and two aspartate residues (EDD) at position 370–372 and a glutamate at position 392 (Fig. 2).

The deduced amino acid sequence of *Xenopus laevis* calreticulin displays 76% similarity to that of rabbit skeletal muscle and mouse liver calreticulin [3,5]; the differences are mainly located at the *N*- and *C*-termini, whereas the proline-rich domain is more conserved. Altogether the percentage of charged residues of the two molecules is similar: 14.5 as opposed to 13.6% positively charged amino acids and 26.3% as opposed to 27.8% negatively charged residues in rabbit skeletal muscle and *Xenopus laevis* brain calreticulin respectively. Like mammalian calreticulin, the amphibian protein has a glycosylation consensus sequence preceding the *C*-terminal acidic domain (residue 339, clone 3; residue 316, clone 8; boxed sequence in Fig. 2).

DISCUSSION

Calreticulin is a microsomal low-affinity high-capacity Ca^{2+}-binding protein [1–5,12]. In view of its possible involvement in intracellular Ca^{2+} storage, calreticulin has attracted a great deal of interest, and a number of studies have been carried out to define its Ca^{2+}-binding characteristics, subcellular distribution and primary structure [1–5,13,14].

High-capacity low-affinity Ca^{2+}-binding proteins appear to be quite heterogeneous in the central nervous system. As part of a comprehensive study on Ca^{2+}-storage proteins, we report for the first time the cloning and sequencing of two calreticulin isoforms. Overall the deduced amino acid sequence of the *Xenopus laevis* calreticulins is 76% similar to that of mouse liver [3] and rabbit skeletal muscle [5]. The amphibian and mammalian proteins show important similarities: (i) the number of negatively charged amino acids is very similar; (ii) they contain a central proline-rich region with internally repeated domains (see [3]); (iii) the glycosylation consensus signal is retained by the amphibian proteins; the conservation of such a site over millions of years of evolution suggests that it may be relevant to protein function and, indeed, at least in the liver, calreticulin is glycosylated [2,15]; (iv) the *C*-terminus is composed of a stretch (38, clone 3; 34, clone 8; and 36, mouse liver) of acidic amino acids, followed by the endoplasmic-reticulum retention signal KDEL [16]. This stretch of negatively charged residues is a characteristic of calreticulin and is believed to be responsible for most of the Ca^{2+}-binding capacity. In fact, Baksh & Michalak recently determined the low- and high-affinity Ca^{2+}-binding domains of mammalian skeletal-muscle calreticulin [13]. They showed that the proline-rich domain of calreticulin displays high-affinity Ca^{2+}-binding sites, whereas the acidic tail binds Ca^{2+} with low affinity. A stretch of acidic residues is also present at the *C*-terminus of calsequestrin, the low-affinity high-capacity Ca^{2+}-binding protein found in the sarcoplasmic reticulum of skeletal muscle [17,18]. Thus the calreticulins expressed in *Xenopus laevis* central nervous system have the fundamental characteristic of Ca^{2+}-storage proteins, namely an acidic tail capable of binding Ca^{2+} with low affinity. Little is known at present about the proteins interacting with calreticulin, and the mechanism by which calreticulin releases its bound Ca^{2+}.

The subcellular localization of calreticulin is still debated [2,14,15]; the presence of the endoplasmic-reticulum retention signal KDEL at the *C*-terminus has been taken as evidence that the protein is homogeneously distributed in endoplasmic-reticulum cisternae. On the contrary, high-resolution immunohistochemical localization in liver has suggested that calreticulin, though present in the endoplasmic reticulum, is concentrated in structures (calciosomes) whose relationship with classical endoplasmic-reticulum cisternae is still unclear [2]. In this context it should be mentioned that the situation in the central nervous system is particularly complex; here not only functionally different Ca^{2+} stores have been identified and characterized [19,20], but also the expression of proteins involved in Ca^{2+} homoeostasis reaches its maximal complexity. In particular, although calreticulin is abundant in the central nervous system [2,5], rat cerebellum Purkinje neurons, the best characterized cells in this context, apparently express an immunologically distinct protein [21]. On the other hand, the same cells in chick cerebellum express a protein which appears to be indistinguishable from skeletal-muscle calsequestrin [22]. Furthermore, several isoforms of the Ca^{2+}-ATPase and of the $InsP_3$ receptor have been cloned and found to be heterogeneously expressed by cells of the central nervous system [23–27]. Immunohistochemical studies have shown that, in addition to $Ins(1,4,5)P_3$-gated Ca^{2+}-release channels, the central nervous system also expresses the ryanodine receptor, the Ca^{2+}-release channel of skeletal muscle [28–31]. The presence of distinct intracellular Ca^{2+} stores is consistent with the existence of multiple calreticulin isoforms. As far as the two calreticulin clones we sequenced are concerned, we do not know whether the proteins they encode are localized in separate compartments within the same cell. *In situ* hybridization experiments will be required to determine whether the different isoforms of calreticulin occupy distinct regions of the central nervous system.

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