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Short communication

Molecular cloning and characterization of sea bass (*Dicentrarchus labrax*, L.) calreticulin

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ABSTRACT

Mammalian calreticulin (CRT) is a key molecular chaperone and regulator of Ca^{2+} homeostasis in endoplasmic reticulum (ER), also being implicated in a variety of physiological/pathological processes outside the ER. Importantly, it is involved in assembly of MHC class I molecules. In this work, sea bass (*Dicentrarchus labrax*) CRT (Dila-CRT) gene and cDNA have been isolated and characterized. The mature protein retains two conserved motifs, three structural/functional domains (N, P and C), three type 1 and 2 motifs repeated in tandem, a conserved pair of cysteines and ER-retention motif. It is a single-copy gene composed of 9 exons. Dila-CRT three-dimensional homology models are consistent with the structural features described for mammalian molecules. Together, these results are supportive of a highly conserved structure of CRT through evolution. Moreover, the present data provides information that will allow further studies on sea bass CRT involvement in immunity and in particular class I antigen presentation.

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1. Introduction

Calreticulin (CALR or CRT) was first identified as a high-affinity Ca^{2+}-binding protein in the sarcoplasmic reticulum of skeletal muscle [1]. Different designations have been attributed to it, but the accepted term calreticulin reflects both its calcium binding nature and its localization in the sarcoplasmic/endoplasmic reticulum (SR/ER) of muscular/non-muscular tissues, respectively (reviewed in [2]). Being present in vertebrates, invertebrates and higher plants (reviewed in [3]), its importance has been demonstrated by multiple functions both inside (chaperoning and Ca^{2+} storage/release) and outside the ER (many physiological/pathological processes) (reviewed in [4,5]).

Calreticulin can be divided into three domains (reviewed in [6]): the amino-terminal N domain, the flexible mid proline-rich P domain, both taking part in chaperone activity, and the highly acidic carboxyl-terminal C domain responsible for the Ca^{2+}-buffering activity (reviewed in [5,6]) that ends with the ER-retrieval sequence [7]. The overall shape of CRT includes a globular structure (N/C domains) [8–10] and an extended arm-like hairpin structure (P domain) [11]. Besides Ca^{2+} [12–14], CRT also binds ATP [15] and Zn^{2+} [16], which affect its ability to suppress aggregation of non-glycosylated proteins [17]. Hence, CRT acts as a lectin chaperone (interacts with monoglycosylated proteins) and as a classical chaperone (has polypeptide-binding capacity/senses a protein conformational state) [17].

Of notice is the involvement of CRT in major histocompatibility complex (MHC) class I folding and high affinity peptide loading (reviewed in [18,19]). Importantly, assembly occurs within the ER (reviewed in [20,21]) and is dependent on CRT [22].

Studies of calreticulin in teleost fish are still limited to a few species [23–27]. Here we report the identification and characterization of calreticulin from European sea bass, a relevant species for the Mediterranean aquaculture industry. Full cDNA and gene sequences as well as gene copy number have been determined. Moreover, *in silico* structural (primary sequence and 3D homology modelling) and phylogenetic analyses have been performed in order to further characterize this molecule.

2. Materials and methods

2.1. Fish

Sea bass, *D. labrax*, were kept in a recirculating, ozone-treated salt-water (20–25‰) system at 22 ± 1 °C and fed with commercial pellets twice a day. Fish were sacrificed with a lethal dose of 2-phenoxyethanol (Panreac; >5 mL/10 L).

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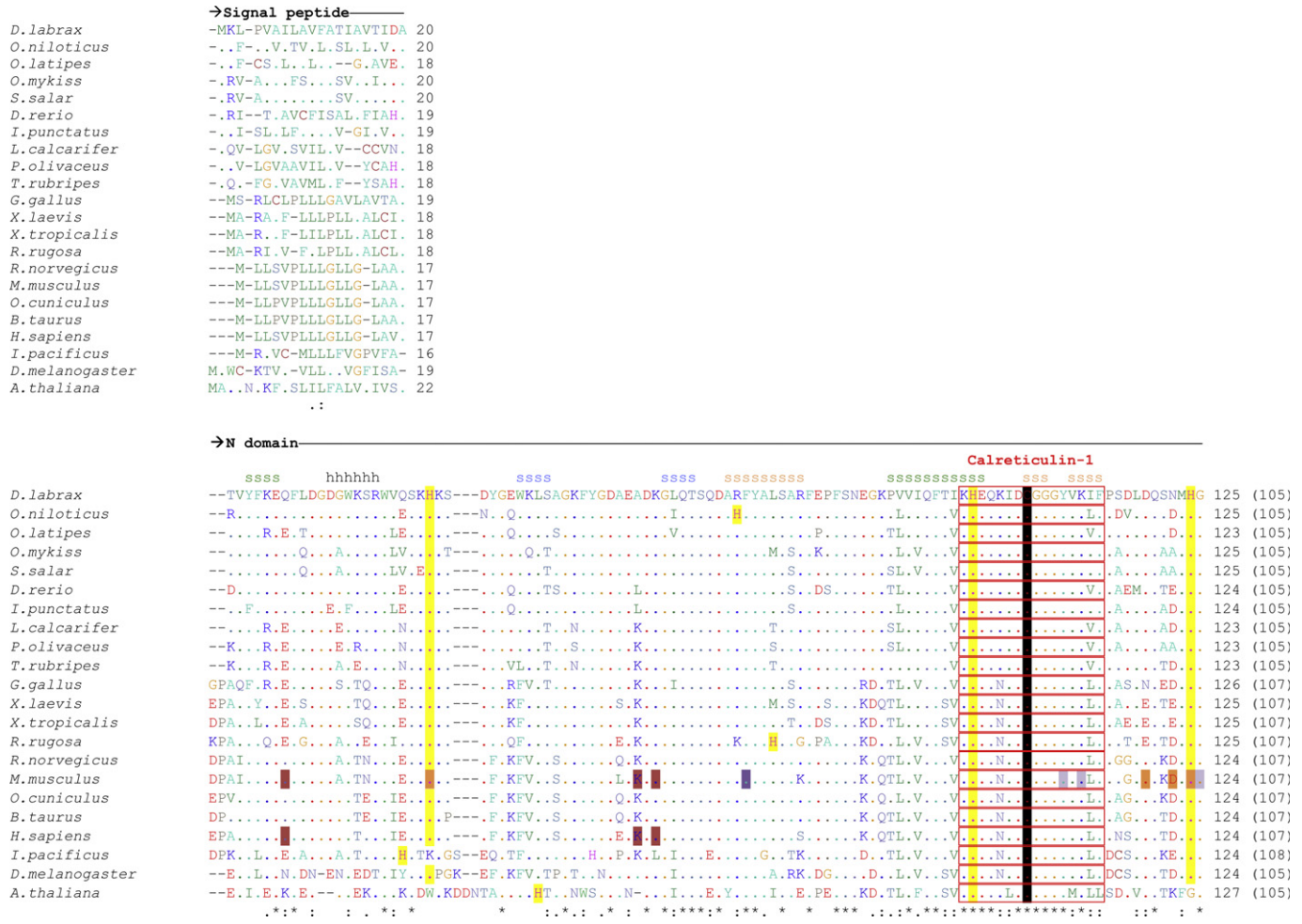

Fig. 1. Alignment of CRT amino acid sequences. Amino acid sequences were retrieved from GenBank (Table S2) and aligned with CLUSTALW [50]. Dashes indicate gaps that maximize the alignment and dots denote residues identical to the first sequence of the alignment. Identical residues, conserved and semi-conserved substitutions are denoted below the alignment with, (*), (:) and (.), respectively. Residue numbers are given right of the sequences; numbers in parenthesis refer to the mature proteins. Predicted N, P and C domains are indicated above the alignment [10], with junction regions represented by black boxes (open ones along the alignment). Letters (s) or (h) above the alignment denote β-strands or α-helices, respectively; different β-sheets within the globular domain are denoted with different colours. The two CRT signature motifs within the N domain are boxed and labelled (Calreticulin-1 and -2). Type 1 (or A) and 2 (or B) tandem repeats are indicated above the alignment (also underlined and in bold type in the sea bass sequence). Putative nuclear localization signal is shaded in grey. The region interacting with rheumatoid arthritis shared epitope is shaded cyan in the rabbit sequence [46]. Residues of human CRT that contact ERp57 [43] are shaded green. Canonical cysteines involved in an intra-chain disulfide bond (connecting β6 and β7) as well as a third cysteine (absent in *Rana rugosa*) are displayed in inverted type. Histidines within the N domain (putative sites for Zn^{2+} binding) are shaded in yellow. Mouse residues that can putatively bind Zn^{2+} are shaded orange [9]. Putative N-glycosylation signal identified in some species is shaded dark blue. Mouse and human CRT residues known to interact with Ca^{2+} in the globular domain are shaded dark red [8,9]. Residues involved in carbohydrate binding are shaded light (hydrogen bonds) and dark (hydrophobic interactions) purple [9]. Hydrophobic patch on the P domain putatively involved in peptide binding is shaded red [10]. Residues involved in the formation of three small hydrophobic cores in the rat P domain are underlined and in bold type [11]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. cDNA cloning

RNAs were extracted according to Versagene™ RNA tissue kit (Gentra Systems) instructions and transcribed to cDNAs following the BioScript RNase H^- (Bioline) protocol. PCR products were cloned into pGEM®-T Easy (Promega). Automated sequencing was performed using primers detailed in Supplementary Table S1. 5′ RACE experiments were performed with the 5′ RACE System from Invitrogen (Version 2.0) and Recombinant Terminal Transferase (Fermentas or Roche) was used to dATP tail the purified cDNA.

Total RNA extracted from the head-kidney of one fish was reverse transcribed with primer APv2 (Table S1). Degenerate primers were designed based on conserved regions of calreticulin across several vertebrate species. The cDNA was amplified with primers CnxCrtFW1 and CnxCrtRV1 (Table S1) and products were obtained at the 4th round of PCR. A product of approximately 400 bp was purified, cloned and sequenced.

This first product from sea bass calreticulin was used to design specific reverse primers. As the distance to the 5′ end of the transcript was still considerable, also a degenerate forward primer was designed using the calnexin/calreticulin multiple alignment. Total RNA from head-kidney was synthesized with APv2 and amplified with the set of primers CrtFw1/DLCrtRV5 followed by two consecutive amplifications with CrtFW1/DLCrtRV4 (Table S1). The obtained ~350 bp product was cloned and sequenced.

To obtain the 5′ UTR of calreticulin, the resulting sequence was used to design two specific reverse primers (Table S1). Total RNA extracted from the spleen of one fish was synthesized with DLCrtRV7. The tailed cDNA was amplified with primers APv2/DLCrtRV7 followed by a 2nd amplification with AUAP2/DLCrtRV6 (Table S1). The obtained ~400 bp product was purified, cloned and sequenced.

To obtain the full sea bass calreticulin cDNA, specific primers (Table S1) were designed at the beginning of the 5′ UTR region.

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