Calreticulin: a Functional Analogue of Calsequestrin?

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Abstract

Calreticulin and calsequestrin are the major Ca^{2+} binding proteins present in the lumen of the sarcoplasmic/endoplasmic reticulum (SR/ER) membranes. Calsequestrin is a major component of skeletal and cardiac muscle SR whereas calreticulin is a minor component in these membranes. However, calreticulin is also found in a variety of non-muscle tissues and in smooth muscle cells. Both calreticulin and calsequestrin bind Ca^{2+} with a low affinity and high capacity. Calreticulin also contains a high affinity, low capacity Ca^{2+} binding site which is not found in calsequestrin and it is not of an EF-hand like structure. Both proteins share several other "diagnostic" biochemical properties, including staining blue with the cationic carbocyanine dye "Stains-All", and having an electrophoretic mobility on SDS-PAGE which is highly pH-sensitive. Despite these apparent "biochemical" similarities the amino acid sequence information and tissue distribution of these two Ca^{2+} binding proteins indicate clearly that calreticulin and calsequestrin are quite different proteins. The only similarity in the amino acid sequences of these proteins occurs at the carboxyl-terminal, where clusters of acidic residues are found in both proteins. These are probably involved in the high capacity Ca^{2+} binding observed in both molecules. Structurally these two proteins also have very little in common.

In this paper we review some of the recent evidence concerning identification and tissue specific localization of calreticulin, calsequestrin and calsequestrin-like proteins. On the basis of this evidence and the structural/functional analysis of calsequestrin and calreticulin undertaken here, we propose that calreticulin (an ER membrane specific protein) might be a functional analog of calsequestrin (an SR membrane specific protein). However, we emphasize that despite this putative shared functional role calsequestrin and calreticulin are in fact quite different proteins.

Key words: calreticulin, calsequestrin, calcium binding proteins, sarcoplasmic reticulum, endoplasmic reticulum, calcium storage

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Ca^{2+} is the second messenger for a wide variety of cellular functions, including the regulation of metabolic pathways, the synthesis and release of hormones, muscle and non-muscle motility, and several membrane linked processes [4]. Most living cells, including muscle, maintain cytoplasmic Ca^{2+} at submicromolar levels, against steep concentration gradients both at the cell surface and across the endoplasmic/sarcoplasmic reticulum (ER/SR) [4]. In striated muscle intracellular concentrations of Ca^{2+} are regulated by the proteins of the SR [10]. In smooth muscle cells the SR has also been clearly identified as the intracellular organelle storing Ca^{2+} [44, 49]. In non-muscle cells evidence now indicates that the ER is responsible for the regulation of cytosolic Ca^{2+} concentrations [54], and it is becoming apparent that the movement of Ca^{2+} ions to and from the ER is regulated by a series of proteins which are analogous to the proteins of striated and smooth muscle SR [49]. For example, whilst the mechanisms of Ca^{2+} release in these systems differ, both involve similar high molecular weight, integral membrane proteins (the $InsP_3$ receptor and the ryanodine receptor, respectively), which function as Ca^{2+} channels. The primary structures of the ryanodine receptor [57] and the $InsP_3$ (inositol 1, 4, 5 - triphosphate) receptor [17, 33] have recently been reported and reveal that these proteins have significant structural similarities. Interestingly, functional evidence suggests that in smooth muscle SR these two different Ca^{2+} release channels are co-expressed [44, 49].

Ca^{2+} is actively taken up from the cytosol, by the action of Ca^{2+}-Mg^{2+} dependent ATPases, into the SR/ER where it must then be buffered within the lumen of these membranes. This buffering is required in order to reduce the concentration gradient against which the Ca^{2+}-ATPase must pump and in muscle SR it is also known to enhance the storage of Ca^{2+} near to the Ca^{2+} release channels. Within the SR of striated muscle this buffering of Ca^{2+} is achieved by calsequestrin, a low affinity, high capacity Ca^{2+} binding protein which is localized specifically to the lumen of the junctional SR [27]. Alongside calsequestrin, striated muscle also contains other Ca^{2+} binding proteins; in particular, it contains calreticulin (earlier referred to as the high affinity Ca^{2+} binding protein) [40] which is also localized within the lumen of the SR [31]. In contrast to striated muscle, the precise identity of Ca^{2+}

binding proteins in the lumen of the ER/SR of smooth muscle and non-muscle tissues has recently been the subject of much controversy. Calsequestrin, calreticulin and even "calsequestrin-like" proteins have all been reported to be possible candidates for Ca^{2+} binding and storage proteins within the lumen of the SR/ER membranes of these tissues [8, 12, 18, 23, 34, 46, 52]. However, we recently clearly identified calreticulin in a variety of non-muscle tissues [12], and the amino acid sequence of calreticulin revealed that the same protein has been independently identified in non-muscle tissues by others, but was referred to as calregulin [53].

The purpose of this review is to compare the structure/function relationships of calsequestrin and calreticulin, and to discuss their potential physiological roles in relation to evidence concerning their localization. In view of steadily accumulating evidence that calreticulin, rather than calsequestrin or "calsequestrin-like" proteins, is a major Ca^{2+} binding protein in the ER/SR of non-muscle and smooth muscle tissues, consideration is given to the possibility that there may be some functional analogy between the two proteins.

As far as is possible, throughout this article we have tried to refer to existing review articles covering aspects of cellular Ca^{2+} homeostasis and the structure and function of Ca^{2+} binding proteins. We have, therefore, undoubtedly introduced some unintentional omissions in our literature citations, and we apologize for these.

Calsequestrin

Calsequestrin is the major Ca^{2+} binding protein in the SR of both cardiac and skeletal muscle, and is known to be involved in the sequestration of Ca^{2+} (for review see ref. 27). Calsequestrin is specifically localized to the terminal cisternae of the SR, where it is observed as an electron-dense matrix [27]. It thus functions to localize Ca^{2+} near to the junctional face membrane and to the Ca^{2+} release channels. The electron dense matrix of calsequestrin appears to be tightly linked to the SR membrane within the terminal cisternae [27], and recently Jones' group [35] has identified a putative calsequestrin binding protein in this region of the SR. In addition, Ikemoto's group [20] has proposed that calsequestrin has some direct interaction with the ryanodine receptor.

Extensive studies have been carried out to determine the physicochemical properties of calsequestrin. It is highly acidic and binds Ca^{2+} with a high capacity (about 40-50 moles of Ca^{2+}/mol of protein) and a moderate to low affinity (K_d=1 mM). As a result of these properties calsequestrin exhibits several other "diagnostic" biochemical properties, including staining blue with the cationic carbocyanine dye "Stains-All", and having an electrophoretic mobility on SDS-PAGE which is highly pH-sensitive [12, 27, 31]. Ca^{2+}-induced changes in the conformation of calsequestrin have been postulated on the basis of a number of studies which have included tryptophan fluorescence, CD, Raman spectroscopy and ^1H NMR, and proteolytic digestion [27].

Calreticulin

Calreticulin is found in skeletal muscle SR membranes alongside calsequestrin, but in several fold lower concentrations than calsequestrin [31]. It was initially identified in these membranes as the high affinity Ca^{2+}-binding protein [40], and recently, using several independent biochemical techniques, it has been specifically localized to the lumen of the SR [11, 31, 34]. Calreticulin is now also known to be present in a variety of non-muscle tissues [13, 34], and has recently been shown to be a major Ca^{2+}-binding protein of both liver ER and smooth muscle SR [34]. Like calsequestrin, calreticulin binds Ca^{2+} with a low affinity and high capacity (K_d=~250 μM, B_{max}=25 moles of Ca^{2+}/mole of protein [40]. However, it also exhibits a single high-affinity binding site, K_d=~1 μM, B_{max}=1 mole of Ca^{2+}/mole of protein) [40]. Again, like calsequestrin, calreticulin has a low pI, and this in conjunction with its Ca^{2+} binding properties is probably responsible for the other biochemical properties it has in common with calsequestrin, namely that it stains blue with "Stains-All", and has a pH sensitive electrophoretic mobility on SDS-PAGE.

The amino acid sequence of calreticulin was recently obtained in our laboratory, from its cDNA, and was used to establish that it is identical to CRP55 and to calregulin, two Ca^{2+}-binding proteins previously identified in rat and bovine liver ER membranes [34, 46, 53]. In addition to binding Ca^{2+}, calreticulin (calregulin) has also been shown to bind Zn^{2+} with a high capacity (14 moles/mole protein) and low affinity [22].

Calreticulin and calsequestrin have different amino acid sequences

A cDNA encoding calreticulin was isolated in our laboratory, from a rabbit skeletal muscle cDNA library, and the amino acid sequence of the protein was deduced from its nucleotide sequence [11]. More recently, mouse, rat, Drosophila and human forms of calreticulin have been cloned [28, 29, 37, 46]. Preliminary Southern blot analysis of human genomic DNA indicates that the calreticulin gene is not highly polymorphic, and exists as a single copy occupying ~ 6kb of genomic DNA [28]. Using human calreticulin cDNA the gene was recently localized to (human) chromosome 19 [28]. Two different isoforms of calsequestrin, skeletal and cardiac, have been identified, both of which have now been characterized and cloned [14, 45]. The gene encoding skeletal calsequestrin is much larger than that for calreticulin, and has been shown to be divided into 11 exons and to span about 14kb of genomic DNA [56]. This gene has been localized to chromosome 1 [16].

Figure 1 shows the deduced amino acid sequences of calreticulin [11], cardiac muscle calsequestrin [45] and skeletal muscle calsequestrin [14]. The overall identity between the two forms of calsequestrin is approximately 60%. However, despite the several biochemical properties that calsequestrin and calreticulin have in common, calreticulin shows no apparent similarity (or identity) to either of the two forms of calsequestrin, except for a similar distribution of negatively charged amino acids at the C-terminal end of the molecule (Fig. 1). In fact, the overall identity between the proteins is less than 10%. Further, even within the acidic C-terminal regions close examination reveals additional significant differences. For example, whilst the last 50 amino acids in all three proteins are highly acidic, in the case of the two isoforms of calsequestrin this region consists of long stretches of aspartic and glutamic acid residues; in calreticulin the acidic residues in this region of the molecule are interspersed at regular intervals with one or more positively charged residues (Fig.1). This difference might lead to different folding patterns for this part of the molecule in calreticulin compared with calsequestrin. Despite these obvious differences in the amino acid sequences of calreticulin and calsequestrin the

overall amino acid composition of the proteins is sufficiently similar that, as discussed above, they can be identified by a number of common biochemical properties which reflect their shared Ca^{2+} binding behavior and acidic pI [11, 24, 27, 31, 34].

Calsequestrin is known to be a glycoprotein which contains NH_2-linked high mannose sugars [47] and binds Concanavalin A [27]. Sequence analysis reveals one potential glycosylation site in calreticulin (residue 326)[11], but we have failed to detect any carbohydrate attached to the skeletal muscle and pancreatic protein (Baksh and Michalak, unpublished observation). In contrast, other evidence indicates that bovine calreticulin (calregulin) is glycosylated, as a result of which it binds to Concanavalin A-Sepharose [21]. In addition, rat liver calreticulin [48] has been postulated to contain a complex hybrid type of oligosaccharide with a terminal galactose residue. This latter type of glycosylation is very unusual for ER proteins, and therefore, at present it appears that further studies are required to firmly establish the glycosylation pattern of calreticulin. Cala and Jones [3] have recently shown that calsequestrin is a very good substrate for casein kinase II. Interestingly, a number of possible casein kinase II phosphorylation sites are also found in the sequence of calreticulin [11, 46]. However, whether or not calreticulin is phosphorylated either *in vitro* or *in vivo* remains to be determined.

Calreticulin terminates with the KDEL sequence [11, 28, 37, 46]. This sequence is known to be responsible for the retention of newly synthesized proteins within the lumen of the ER [41]. A putative KDEL receptor was recently identified using anti-idiotypic antibodies [50] and it was postulated that Ca^{2+} binding to KDEL proteins may be an important regulatory factor in their interactions with this receptor. Since calreticulin contains this sequence it is most likely salvaged as an ER/SR protein, supporting our earlier observation that calreticulin is localized to the lumen of the SR/ER [31]. In contrast, calsequestrin does not terminate with the KDEL sequence. The mechanism(s) responsible for the localization and retention of calsequestrin in the lumen of the SR are not clear yet. However, since the SR membrane does not perform a secretory function, it is probable that there is no need for a specific retention system for proteins within its lumen.

Structure of calreticulin and calsequestrin

Analysis of the amino acid sequence of *calsequestrin* indicates some possible division of the protein into distinct structural domains. This suggestion is supported by recent evidence which indicates some localization of different functions of the protein into different regions of its sequence. Particularly, a region of the protein which encompasses amino acids 80 - 100 has been suggested to interact with the ryanodine receptor/Ca^{2+} release channel [20]. In this study Collins *et al.* [6] identified several sequences (KLGLTEEDSI; LGLYEEDSIY; EDVIEYDGE) within this region of the protein which might be involved in its interaction with the ryanodine receptor. Analysis of the amino acid sequence of calsequestrin indicates that residues 150-300 form a region particularly rich in α-helices [14, 45], whilst amino acids 300-367 are exceptionally rich in the acidic amino acids, and form a C-terminal acidic tail domain.

Calreticulin, similar to calsequestrin, is synthesized with a signal sequence [11, 14, 45, 46]. Secondary structure predictions for this protein suggest that it might have a "lollipop" shape which can be clearly divided into structurally distinct domains [11, 46]. The mature protein sequence begins with a helix-turn-helix motif followed by a sequence predicted to form 8 anti-parallel-strands connected by protein loops (*β-sheets domain*). The neighbouring region of calreticulin contains an abundance of proline residues, some of which are spaced regularly every 4 or 5 amino acids (*P-rich domain*), and within this region the sequence KPEDWD is repeated three times. This particular portion of the protein sequence also contains a putative nuclear targeting signal, PPKKIKDPD, which could possibly facilitate transport of calreticulin into the nucleus. This domain is predicted to have a repeating, rigid turn structure, which separates the globular head of the protein from the *acidic tail-domain.* This region is also highly charged. Finally, the C-terminal 20% of calreticulin is highly acidic (*acidic tail-domain*); in the last 56 residues, 37 are acidic, while 10 are basic.

Ca^{2+} binding sites on calsequestrin and calreticulin

Calreticulin has a single high affinity Ca^{2+}-binding site along with a number of low affinity sites [40]. Despite the presence of this high-affinity Ca^{2+} binding site on calreticulin no evidence for an EF hand consensus sequence is observed in its primary structure [11, 46]. As a result of this, the location of the high affinity Ca^{2+}-binding site is currently unknown. However, our preliminary experiments suggest that this binding is localized to the *P-rich domain* of calreticulin (Baksh and Michalak, unpublished observations). Further identification and characterization of this high affinity Ca^{2+}-binding site in calreticulin may contribute to our understanding of Ca^{2+}-binding domains in other proteins. The sequence of the *acidic tail-domain* of calreticulin [11] is similar to the acidic C-terminal region found in calsequestrin [14, 45], a protein which is known to bind up to 40 moles of Ca^{2+} per mole [27]. The acidic C-terminal end of calsequestrin has been shown to be involved in Ca^{2+} binding to the protein [38]. On the basis of this similarity it is probable that the highly acidic region of calreticulin is responsible for its low affinity Ca^{2+} binding properties. This suggestion is supported by observation of similar clusters of acidic amino acid residues in other Ca^{2+} binding proteins, including the Ca^{2+}-binding 160-kDa SR membrane glycoprotein (sarcalumenin) [25], the 165-kDa HCP, LDL and Ca^{2+}-binding protein of SR [19], the ryanodine receptor [57], the major human centromere autoantigen CENP-B [9] and some heat shock proteins [13]. Our preliminary studies with fusion proteins encoding portions of the calreticulin molecule also support this suggestion, since they indicate that the *acidic tail-domain* of calreticulin does bind Ca^{2+} with a low affinity and high capacity (Baksh and Michalak, unpublished observations).

The CD and absorption spectra of calreticulin do not change significantly upon Ca^{2+} binding (22, 39, Baksh, Kay and Michalak, unpublished observations). This contrasts with observations for calsequestrin, which is known to undergo marked conformational changes upon the binding of Ca^{2+} [27]. However, studies by Waisman's group [22] have shown that Ca^{2+} binding to calreticulin may result in a modest increase in its intrinsic fluorescence. The same group has shown that calreticulin (calregulin) is also a Zn^{2+} binding protein, and that the binding of Zn^{2+} causes a dramatic increase in its intrinsic fluorescence [22]. These observations suggest that Zn^{2+} binding to the protein causes the exposure

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                10         20         30         40         50         60         70         80
CRT - EPVVYFKEQF LDGDGWTERW IESKHKSDFG KFVLSSGKFY GDQEKDKGLQ TSQDARFYAL SARFEPFSNK GQPLVVQFTV
      *    *               *                    *    *         *  *         *   *
CCS - EEGLNFPTYD GKDRVVSLTE KNFKQVLKKY DVLCLYYHES VSSDKVAQKQ FQLKEIVLEL VAQVLEHKDI GFVMVDAKKE
      **** ** ** * ***      ** * * ***  ** * ***    **  * * *   *  ***  ***** *   **  **  *
SCS - EEGLDFPEYD GVDRVINVNA KNYKNVFKKY EVLALLYHEP PEDDKASQRQ FEMEELILEL AAQVLEDKGV GFGLVDSEKD

                90         100        110        120        130        140        150        160
CRT - KHEQNIDCGG GYVKLFPAGL DQKDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR
                     *          *                    *  *                    *    *
CCS - AKLAKKLGFD EEGSLYVLKG DRTIEFDGEF AADVLVEFLL DLIEDPVEII NSKLEVQAFE RIEDQIKLIG FFKSEESEYY
      *  *****   ** * ** *  *  ** ****  ** ****** *  ***** *      * ****  *** *****  **   ** *
SCS - AAVAKKLGLT EEDSIYVFKE DEVIEYDGEF SADTLVEFLL DVLEDPVELI EGERELQAFE NIEDEIKLIG YFKNKDSEHY

                170        180        190        200        210        220        230        240
CRT - PDNTYEVKID NSQVESGSLE DDWDFLPPKK IKDPDASKPE DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM
                           *                       **   * *       *          *          *
CCS - KAFEEAAEHF QPYIKFFATF DKGVAKKLSL KMNEVDFYEP FMDEPIAIPD KPYTEEELVE FVKEHQRPTL RRLRPEDMFE
      *** **** *  *** ***** *  ***** * * ** ****  ** **  *** **  *** *  ** ** * ** * * ** * *
SCS - KAFKEAAEEF HPYIPFFATF DSKVAKKLTL KLNEIDFYEA FMEEPVTIPD KPNSEEEIVN FVEEHRRSTL RKLKPESMYE

                250        260        270        280        290        300        310        320
CRT - DGEWEPPVIQ NPEYKGEWKP RQIDNPDYKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD LWQVKSGTIF DNFLITNDEA
        *     *            *                    ***   *            *                        *
CCS - TWEDDLNGIH IVAFAERSDP DGYEFLEILK QVARDNTDNP DLSIVWIDPD DFPLLVAYWE KTFKIDLFKP QIGVVNVTDA
      ****** *** ******  ** **********  ** ****** **** ***** ****** *** *** ***  * **********
SCS - TWEDDMDGIH IVAFAEEADP DGYEFLEILK SVAQDNTDNP DLSIIWIDPD DFPLLVPYWE KTFDIDLSAP QIGVVNVTDA

                330        340        350        360        370        380        390        401
CRT - YAEEFGNETW GVTKTAEKQM KDKQDEEQRL KEEEEEKKRK EEEEAEEDEE DKDDKEDEDE DEEDKDEEEE EAAAGQAKDEL
                     ***        *          *                 * **    **    *        *
CCS - DSVWMEIPDD DDLPTAEELE DWIEDVLSGK INTEDDDNEE GDDGDDDEDD DDDDGNNSDE ESNDDSDDDD E
      *** **  *   *** ***** ** **** *  ******* *   ** ***
SCS - DSVMMEMDDE EDLPSAEELE DWLEDVLEGE INTEDDDDED DDDDDDD
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Figure 1. Amino acid sequences of skeletal muscle calreticulin (CRT), cardiac calsequestrin (CCS) and skeletal muscle calsequestrin (SCS).
The amino acid sequences of calreticulin and calsequestrins were deduced from the nucleotide sequences of cDNA encoding these proteins and are taken from earlier reports [11, 14, 45]. The regions of perfect identity between the three sequences are represented by ""*

of a domain with considerable hydrophobic character. Interestingly, the primary structure of calreticulin contains no sequences which resemble the consensus sequence for a zinc-finger [11]. Our preliminary experiments suggest that, like the high affinity Ca^{2+} binding site, the Zn^{2+} binding sites in calreticulin are localized to the *P-rich domain* (Baksh and Michalak, unpublished observations).

The tissue distribution of calsequestrin and calreticulin

Calsequestrin has been specifically localized to the lumen of the junctional SR in both skeletal and cardiac muscle [27]. Calreticulin has also been localized to the SR in these tissues [31]. In contrast, there has been a great deal of confusion regarding the identity of Ca^{2+} binding proteins in non-muscle and smooth muscle tissues. A major contributing factor in this confusion has been the frequent mis-identification of calreticulin as a "calsequestrin-like" protein. For example, a "calsequestrin-like" protein reported in liver [8] was found to have an NH_2-terminal amino acid sequence identical to calreticulin [34]. The same was found for a canine brain Ca^{2+}-binding protein [7]. A major Ca^{2+}-binding protein identified in HL-60 cells and referred to as a "calsequestrin-like" protein was also recently found to have the same NH_2-terminal amino acid sequence as calreticulin [24]. This confusion concerning the identities of calreticulin and calsequestrin in non-muscle systems has occurred primarily because of the shared biochemical properties of the two proteins, which, as described earlier, are a function of their Ca^{2+} binding behavior. In addition, many antisera produced against calsequestrin unfortunately also cross-react with calreticulin. This may be due to some similarity in the amino acid sequences of the proteins, particularly within the acidic C-terminal portions of the molecules.

Using immunofluorescence, Western blotting and Northern blotting techniques we have recently shown that calreticulin, and not calsequestrin, is a major Ca^{2+}-binding protein present in isolated liver ER vesicles and in smooth muscle SR vesicles

[34]. Despite several recent reports of calsequestrin being detected in non-muscle tissues [8, 18, 23, 52] we detected neither calsequestrin nor calsequestrin mRNA in non-muscle tissues [34]. Scott *et al.* [45] were also unable to find any evidence for calsequestrin mRNA in non-muscle tissues (canine liver and brain). Using immunological methods very low amounts of calsequestrin (cardiac form) have been found in some smooth muscle cells [22], and Wuytack *et al.* [55] also detected small amounts of calsequestrin in SR vesicles isolated from smooth muscle. This group, however, found several other Ca^{2+} binding proteins in the smooth muscle SR vesicles, a major one of which was calreticulin. Recently, some calsequestrin has also been shown to be present in chicken cerebellum [51].

"Calciosomes" and calsequestrin-like proteins

Along with some confusion regarding the identity of Ca^{2+} binding proteins in non-muscle and smooth muscle tissues there has also been some dispute as to the intracellular localization of these proteins. Particularly, some confusion has surrounded the proposal that calsequestrin or a "calsequestrin-like" protein might be a major component of a newly identified subcellular organelle, the calciosome, which was identified as an $InsP_3$ sensitive Ca^{2+} storage and release organelle of non-muscle systems [8, 18, 23, 52]. We have, firstly, been unable to identify calsequestrin in liver ER [34]. Secondly, we found no evidence for any specialized vesicles containing calreticulin which could be the equivalent of the calsequestrin containing calciosomes [34, Opas and Michalak, submitted for publication]. Indeed, indirect immunofluorescent staining of frozen sections of cultured cells from a variety of tissues shows that calreticulin is localized predominantly to the ER membranes [12]. Similarly, Soling's group [48] was unable to detect calsequestrin in non-muscle tissues, and also found no indication for $InsP_3$-sensitive specialized organelle(s) containing calsequestrin-like protein(s).

Calreticulin and muscle development

The rate of synthesis of calreticulin in differentiating primary cultures of rat skeletal muscle cells is relatively high in myoblasts but declines after cell fusion [32]. Using immunocytochemical methods we have analyzed the intracellular distribution of calreticulin as a function of differentiation in muscle cells. In proliferating (non-muscle) cells, and especially in continuous cell lines, calreticulin is present in large quantities and is distributed throughout the ER. In proliferating muscle cells (myoblasts) calreticulin follows this standard pattern, but with some additional nucleolar staining, the origin of which is unclear to us at present (Opas and Michalak, unpublished observations). If myoblast fusion is inhibited with either high serum concentration, TGF-ß or phorbol esters, the nucleolar staining disappears although the ER localization of calreticulin remains unchanged. In contrast, in differentiated (i.e. fused) muscle cells both the ER and the nucleolar staining for calreticulin are abolished [12]. These data support the suggestion that calreticulin might be down regulated during muscle development. Interestingly, the down-regulation of calreticulin in developing muscle cells correlates with a previously observed [32] up-regulation of the expression of calsequestrin, and it is possible, therefore, that the regulation of the expression of both genes may be by similar mechanisms.

Calreticulin is present in a diversity of organisms

The nucleotide sequence of our cDNA for calreticulin (rabbit) shows over 92% amino acid sequence identity with both CRP55 (mouse calreticulin) and human calreticulin (the Ro/SS-A autoantigen) [11, 28, 29, 34, 46]. This observation indicates marked conservation of the protein in different mammalian species. In addition, other molecules have recently been identified that are highly homologous to calreticulin, in *O. volvulus*, *D. melanogaster* and *A. californica* [29]. NH(2)-terminal amino acid sequence similarities between calreticulin and the *Aplysia* p407 memory molecule suggest that the p407 is an *Aplysia* homologue of calreticulin [29, 34]. The *Aplysia* p407 protein was identified as one of several proteins whose net rate of synthesis is increased during long-term sensitization [5]. Molecular cloning of the p407 molecule has recently confirmed its identity with calreticulin (Drs. T.D. Kennedy and E.R. Kandel, personal communication). This evidence is highly suggestive that calreticulin is present in a wide variety of cellular systems where it possibly performs very basic cellular functions involving Ca^{2+} storage.

Calreticulin in autoimmune diseases

The suggested identity between calreticulin and the Ro/SS-A antigen [28, 29] is clinically relevant since antibodies directed against the Ro/SS-A autoantigen are found in a majority of the patients with primary Sjorgen's Syndrome, subacute cutaneous lupus erythematosus, neonatal lupus erythematosus, anti-nuclear antibody negative lupus erythematosus and systemic lupus erythematosus-like disease secondary to homozygous C2 or C4 complement deficiency. Recently, it has been demonstrated that there are at least four immunologically distinct Ro proteins which react with human Ro antisera, some of which may be localized to the nucleus [43]. Several of these proteins, including calreticulin, form a complex with the hY RNAs [1, 28]. The functional significance of these interactions is not clear.

One potentially severe and permanent manifestation of neonatal lupus is congenital complete heart block (CCHB) which can appear after the first trimester of pregnancy. The mechanism of the disease is thought to involve placental transport of maternal antibodies which initiate specific myocardial inflammation that can permanently damage the conduction system of the developing heart [2]. Antibodies to the Ro antigen have been found in sera from over 85% of mothers of infants with CCHB.

Patients infected with *O. volvulus*, a filarial nematode that causes river blindness, have antibodies directed against the onchocercal RAL-1 antigen. The similarities between calreticulin and this protein are particularly striking in the *ß-sheet* and *P-rich domains*. The area of least homology is the C-terminal region, since the C-terminus of the onchocercal protein is positively rather than negatively charged [29].

Two acidic amino acid sequence repeats in CENP-B, the major human centromere autoantigen, are strikingly similar to the "acidic tail" domain of calreticulin [11]. CENP-B is a major component of the central part of the centromere domain [42]. Centromere components, including CENP-B, are the target of a highly selective autoimmune response in certain patients with rheumatic diseases [15, 36].

The functional significance of calreticulin, and the proteins with homology to calreticulin, in these (auto)immune responses has still to be determined.

Conclusions

Recent evidence, which we have reviewed in this paper, now strongly suggests that calreticulin could be a functional analogue of calsequestrin. Firstly, calreticulin has been shown to be a major Ca^{2+} binding protein in the ER membranes of non-muscle tissues. Secondly, it is known to bind Ca^{2+} with a high capacity and low affinity, in a similar manner to calsequestrin. This possible functional analogy is especially interesting in view of the fact that, structurally, these two proteins have very little in common.

Evidence indicates that calreticulin may be expressed in parallel with the $InsP_3$ receptor, whilst calsequestrin is expressed with the ryanodine receptor. For example, calsequestrin is localized in striated muscle SR, in which the ryanodine receptor acts as the Ca^{2+} release channel. A recent report indicates that some calsequestrin is expressed in the cerebellum [51], and, interestingly, the ryanodine receptor has also been identified in this region of the brain [30]. Most evidence now indicates that both calreticulin and small amounts of calsequestrin are expressed in smooth muscle, and this tissue is known to be sensitive to both ryanodine and $InsP_3$ [44, 49]. Since calsequestrin has been proposed to interact with the ryanodine receptor [20], and in some way affect Ca^{2+} release processes [20], it is possible that calreticulin may have some similar interaction with the $InsP_3$ receptor. Calsequestrin has been shown to be co-localized with the ryanodine receptor specifically in the junctional SR, and, in comparison, it is not impossible that the $InsP_3$ receptor and calreticulin might be co-localized to sub-specialized regions of the ER in non-muscle cells.

In conclusion, it appears that calreticulin might be responsible for Ca^{2+} storage in the lumen of ER membranes. However, despite this suggestion of a shared functional role with calsequestrin, and despite some superficial biochemical similarities between these two Ca^{2+} binding proteins, calsequestrin and calreticulin are in fact quite different. Essentially these two proteins have nothing in common except for the fact that they both bind Ca^{2+}, and it is therefore misleading to refer to calreticulin as a "calsequestrin-like" protein. Calsequestrin appears to be a highly specialized Ca^{2+} binding protein which is present primarily in vertebrate striated muscle. In contrast, calreticulin is a protein with a conserved amino acid sequence which has a widespread distribution throughout the animal kingdom in both muscle and non-muscle tissues.

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