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REVIEW ARTICLE

Calreticulin

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WHAT IS CALRETICULIN?

Calreticulin was first identified many years ago as a Ca^{2+}-binding protein in skeletal muscle sarcoplasmic reticulum (MacLennan *et al.*, 1972; Ostwald & MacLennan, 1974). The protein was observed to bind Ca^{2+} with a high affinity and was consequently named the high-affinity Ca^{2+}-binding protein (Ostwald & MacLennan, 1974). The molecular cloning of calreticulin was only achieved 15 years later, and combined with *N*-terminal amino acid sequence analysis has revealed that this protein has been 'rediscovered' several times by others and described using a variety of different names, including the high-affinity Ca^{2+}-binding protein, calregulin, CRP55 and calsequestrin-like protein (Ostwald & MacLennan, 1974; Waisman *et al.*, 1985; Macer & Koch, 1988; Damiani *et al.*, 1990; Treves *et al.*, 1990). For example, in 1985 Waisman's group (Waisman *et al.*, 1985) identified and isolated a 63 kDa Ca^{2+}-binding protein (CAB-63) from frozen liver and named the protein **calregulin**. Koch's group (Macer & Koch, 1988), while studying Ca^{2+}-binding proteins associated with endoplasmic reticulum membranes, identified a set of proteins that they named reticuloplasmins. One of these proteins was a 55 kDa protein named **CRP55** (calcium binding reticuloplasmin). More recently, Volpe *et al.* (1988) and Damiani *et al.* (1988) identified a Ca^{2+}-binding protein in non-muscle tissues, which was referred to as a '**calsequestrin-like**' protein. Van *et al.* (1989) isolated four Ca^{2+}-binding proteins from rat liver endoplasmic reticulum membranes. One, designated **CaBP3**, has recently been shown to correspond to rat liver calreticulin (Peter *et al.*, 1992). Green's group, while studying the structure and function of the resident proteins of endoplasmic reticulum membranes, has identified, isolated and characterized a number of different proteins including the protein designated **ERp60** (Lewis *et al.*, 1985). The amino acid sequence of ERp60 deduced from its cDNA indicates that it is calreticulin (M. Green, personal communication). It has now been confirmed that calregulin, CRP55, CaBP3, ERp60 and the 'calsequestrin-like' proteins are all, in fact, **calreticulin** (Khanna *et al.*, 1987; Opas *et al.*, 1988, 1991; Fliegel *et al.*, 1989*a*,*b*; Smith & Koch, 1989; Collins *et al.*, 1989; Treves *et al.*, 1990; Krause *et al.*, 1990; Milner *et al.*, 1991; Michalak *et al.*, 1991).

In order to eliminate the confusion regarding the identity of this protein we initially proposed that it be named **reticulin**, reflecting its localization to sarcoplasmic reticulum and endoplasmic reticulum membranes (Opas *et al.*, 1988). Subsequently, following consultation with other laboratories studying this protein, the name **calreticulin** was chosen (**cal**cium binding protein localized to the endoplasmic/sarcoplasmic **reticul**um membranes) (Fliegel *et al.*, 1989*a*; Smith & Koch, 1989). This name is now widely accepted.

It is well established that calreticulin is a high-capacity Ca^{2+}-bindng protein (MacLennan *et al.*, 1972; Treves *et al.*, 1990; Michalak *et al.*, 1991; Baksh & Michalak, 1991). In this review we present evidence which strongly indicates that calreticulin can function as a major Ca^{2+}-binding (storage) protein in the lumen of the endoplasmic reticulum. This is potentially a very important role in the cell, as the storage of Ca^{2+} in the lumen of the endoplasmic or sarcoplasmic reticulum is centrally important to the regulation of cytoplasmic free Ca^{2+} concentrations. Ca^{2+} is taken up from the cytosol by a Ca^{2+}-ATPase, is stored within the membranes and then released, via special channels, upon appropriate stimulation (MacLennan *et al.*, 1983; Carafoli, 1987; MacLennan, 1990; Berridge, 1990; Meldolesi *et al.*, 1990; Pietrobon *et al.*, 1990; Tsien & Tsien, 1990). When 'stored' within the membranes, Ca^{2+} is sequestered at special high-capacity, low-affinity Ca^{2+}-binding (storage) sites. This sequestering is important since it reduces the concentration gradient of Ca^{2+} against which the ATPase must work. A number of different proteins have been implicated to play some role in Ca^{2+} storage (see reviews by Cala *et al.*, 1990; Milner *et al.*, 1992). In the sarcoplasmic reticulum of skeletal and cardiac muscle, calsequestrin has been firmly established to be the major Ca^{2+} storage protein (MacLennan *et al.*, 1983). In contrast, in non-muscle tissues the identity of the Ca^{2+} buffer(s) in the lumen of the endoplasmic reticulum has remained obscure. The recent discovery that calreticulin is a major Ca^{2+}-binding protein of non-muscle endoplasmic reticulum membranes has contributed to the identification and characterization of the Ca^{2+} storage components of endoplasmic reticulum membranes (Opas *et al.*, 1988; Fliegel *et al.*, 1989*b*; Milner *et al.*, 1991).

In addition to its apparent Ca^{2+}-storage role, evidence is rapidly accumulating which suggests that calreticulin has other, perhaps very basic, functions to perform within the cell. This evidence includes: (i) the localization of calreticulin to the nucleus and nuclear envelope, as well as to the endoplasmic reticulum (Opas *et al.*, 1988, 1991; Fliegel *et al.*, 1989*b*); (ii) sequence similarities of calreticulin with a number of other, quite different, cellular proteins (see Fig. 2); (iii) a distinctive domain structure of the protein (Smith & Koch, 1989; Fliegel *et al.*, 1989*a*; Opas *et al.*, 1991; Baksh & Michalak, 1991); (iv) changes in the expression of calreticulin in relation to cellular proliferation and protein synthesis (Gersten *et al.*, 1989, 1991; Opas *et al.*, 1991); (v) direct association of calreticulin with other cellular proteins (Rojiani *et al.*, 1991; Guan *et al.*, 1991); (vi) a proposed role for calreticulin in autoimmune diseases (McCauliffe *et al.*, 1990*a*,*b*; Rokeach *et al.*, 1991*a*). Together these observations, discussed below, suggest that calreticulin is more than just a Ca^{2+}-binding (storage) protein and may well be multifunctional.

CHARACTERISTICS OF CALRETICULIN

Table 1 summarizes the observed physicochemical properties of calreticulin. The molecular mass of calreticulin, based on the amino acid sequence deduced from its cDNA, was estimated to be approx. 46 kDa (Fliegel *et al.*, 1989*a*; Smith & Koch, 1989). However, when analyzed by SDS/PAGE (Laemmli system)

Abbreviations used: Grp, glucose-regulated protein; Hsp, heat-shock protein; BiP, immunoglobulin heavy-chain binding protein.

Table 1. Characteristics of calreticulin

References: 1, Fliegel *et al.* (1989*a*); 2, Smith & Koch (1989); 3, McCauliffe *et al.* (1990*a*); 4, Waisman *et al.* (1985); 5, Treves *et al.* (1990); 6, Milner *et al.* (1991); 7, Baksh & Michalak (1991); 8, Krause *et al.* (1990); 9, Fliegel *et al.* (1989*b*); 10, Van *et al.* (1989); 11, MacLennan *et al.* (1972); 12, Ostwald & MacLennan (1974); 13, Macer & Koch (1988); 14, Michalak *et al.* (1991); 15, Khanna *et al.* (1986).

Molecular mass			
deduced from amino acid sequence		46 kDa	[1–3]
SDS/PAGE (Laemmli system)		60 kDa	[4–7]
sedimentation equilibrium		55 kDa	[4]
pH-dependent change in in apparent molecular mass		Yes	[8]
Stains blue with Stains-All		Yes	[5–9]
Glycosylation*		+/−	[4,10]
Isoelectric point		~4.65	[3,4]
Ca^{2+} binding			[4,5,7,10–14]
high-affinity site (low capacity)	K_d	1.6 μM	
	$B_{max.}$	1 mol/mol of protein	
low-affinity site (high capacity)	K_d	0.3–2 mM	
	$B_{max.}$	20–50 mol/mol of protein	
Zn^{2+} binding			[15]
low-affinity site (high capacity)	K_d	300 μM	
	$B_{max.}$	14 mol/mol of protein	

* Calreticulin has one potential glycosylation site; however, among all of the calreticulins tested so far only the bovine [4] and rat [10] liver proteins have been shown to contain carbohydrate.

calreticulin migrates with an apparent molecular mass of 60000–63000 (Waisman *et al.*, 1985; McCauliffe *et al.*, 1990; Milner *et al.*, 1991). The molecular mass of calreticulin estimated by SDS/PAGE at neutral pH is 55000 (Ostwald & MacLennan, 1974; Michalak *et al.*, 1980) which is similar to the value determined by sedimentation equilibrium, also at neutral pH (Waisman *et al.*, 1985). Discrepancies between predicted and observed mobilities on SDS/PAGE have been reported for several other proteins, including calsequestrin, the 'Ca^{2+}-storage' protein of muscle sarcoplasmic reticulum (MacLennan *et al.*, 1983). Like calsequestrin, calreticulin has a highly charged C-terminal region. The calculated isoelectric point of calreticulin is 4.14 (Fliegel *et al.*, 1989*a*; McCauliffe *et al.*, 1990*a*) and the value measured for the native protein is 4.65–4.67 (Waisman *et al.*, 1985; McCauliffe *et al.*, 1990*a*). This acidity is thought to be responsible for the aberrant migration in gels of both proteins.

It is well documented that calreticulin binds Ca^{2+} (MacLennan *et al.*, 1972; Ostwald & MacLennan, 1974; Waisman *et al.*, 1985; Van *et al.*, 1989; Treves *et al.*, 1990; Milner *et al.*, 1991; Michalak *et al.*, 1991; Baksh & Michalak, 1991) (Table 1). In recent studies, using both native and recombinant calreticulin, it has been demonstrated that this protein contains two distinct types of Ca^{2+}-binding site, one high-affinity/low-capacity and one low-affinity/high-capacity (Baksh & Michalak, 1991). Importantly, these different types of site are located in different regions of the protein (Baksh & Michalak, 1991). These results contrast with other reports of the Ca^{2+}-binding properties of calreticulin. For example, Waisman *et al.* (1985) and Van *et al.* (1989) reported only high-affinity Ca^{2+} binding to calreticulin, whereas Macer & Koch (1988) and Treves *et al.* (1990) showed only low-affinity Ca^{2+} binding to this protein. This discrepancy is most likely due to the different methods used by these authors for the measurement of Ca^{2+} binding. Treves *et al.* (1990) studied Ca^{2+} binding to calreticulin only at relatively high concentrations of Ca^{2+}, and consequently did not detect any high-affinity Ca^{2+} binding. Waisman *et al.* (1985) and Van *et al.* (1989) studied Ca^{2+} binding to calreticulin in the presence of millimolar concentrations of Mg^{2+}. We have found that this protocol reduces Ca^{2+} binding to the high-capacity, low-affinity sites by 60% (Baksh & Michalak, 1991). The Ca^{2+}-binding properties of calreticulin are further discussed below, in relation to the predicted structure of the protein.

In addition to binding Ca^{2+}, calreticulin also binds 14 mol of Zn^{2+}/mol of protein, with relatively low affinity (approx. 300 μM) (Khanna *et al.*, 1986, 1987). This binding of Zn^{2+} to calreticulin induces dramatic conformation changes in the protein which can be measured by changes in its intrinsic fluorescence, its c.d. spectrum, or its interaction with phenyl-Sepharose beads (Khanna *et al.*, 1986; S. Baksh, K. Burns & M. Michalak, unpublished work). This contrasts with Ca^{2+} binding to calreticulin, which does not induce significant conformational changes in the protein (Ostwald *et al.*, 1974; Khanna *et al.*, 1986, 1987; Van *et al.*, 1989; S. Baksh, C. Kay & M. Michalak, unpublished work). The Zn^{2+}-dependent conformational changes appear to involve an increased hydrophobicity of the protein (Khanna *et al.*, 1986). Since calreticulin does not have a 'Zn^{2+}-finger' consensus sequence the location of Zn^{2+} binding to the protein is not clear at present. It is also not known what the functional significance of this Zn^{2+} binding might be. However, the central nervous system contains an abundance of Zn^{2+}, which is localized in the neuronal parenchyma (Frederickson *et al.*, 1987; Frederickson, 1989; Crawford & Connor, 1972). Zn^{2+} is actively taken up (Wolf *et al.*, 1984; Wensink *et al.*, 1988) and stored in large quantities in synaptic vesicles in nerve terminals (Friedman & Price, 1984; Perez-Clausell & Danscher, 1985; Holm *et al.*, 1988) and has been implicated, therefore, to have some neuromodulatory role in synaptic vesicles (Friedman & Price, 1984; Perez-Clausell & Danscher, 1985; Holm *et al.*, 1988; Frederickson, 1989; Xie & Smart, 1991). Interestingly, these vesicles may contain significant amounts of calreticulin (Johnson *et al.*, 1991). Calreticulin might, therefore, play some role in the regulation of both Zn^{2+} and Ca^{2+} levels in synaptic vesicles.

It is currently unclear whether calreticulin is a glycosylated protein. Whilst the sequence contains one potential glycosylation site (residue 326) the search for a carbohydrate moiety has so far been inconclusive. No carbohydrate has been detected in either skeletal muscle or smooth muscle calreticulin (Milner *et al.*, 1991), in human (McCauliffe *et al.*, 1990*a*) or murine (Lewis *et al.*, 1985, 1986) calreticulin. Further, both chicken and rabbit liver calreticulin apparently contain no carbohydrate (Waisman *et al.*, 1985). Despite this, bovine liver calreticulin has been shown to be glycosylated for it binds to Concanavalin A–Sepharose and it is sensitive to endoglycosidase H digestion (Waisman *et al.*, 1985; Lewis *et al.*, 1985). In adddition, it has been shown that rat liver calreticulin contains a complex hybrid type of oligosaccharide with a terminal galactose residue (Van *et al.*, 1989; Peter *et al.*, 1992), a type of glycosylation which is very unusual for resident endoplasmic reticulum proteins. Since the rat liver calreticulin contains a terminal galactose the protein must pass through the *trans*-Golgi before being transported back to the endoplasmic reticulum. Recently, Söling's group have shown that this terminal galactosylation of calreticulin is abolished when the vesicular transport from the intermediate-to-*trans*-Golgi is blocked (Peter *et al.*, 1992). It is postulated that retention of calreticulin mediated by the KDEL receptor must extend into the Golgi, including the *trans*-Golgi (Peter *et al.*,

1992). Further studies are required to establish firmly the glycosylation patterns of calreticulin and the precise trafficking of this protein between different cellular compartments.

A commonly used diagnostic property of calreticulin is that it stains blue with 'Stains-All' (Fliegel *et al.*, 1989*b*; Krause *et al.*, 1990; Treves *et al.*, 1990; Milner *et al.*, 1991; Baksh & Michalak, 1991). This behaviour is similar to that of several other high- and low-affinity Ca^{2+}-binding proteins, including calmodulin, troponin C, S-100, sarcalumenin and calsequestrin (Jones *et al.*, 1979; Campbell *et al.*, 1983).

CELLULAR LOCALIZATION OF CALRETICULIN

As already discussed, calreticulin was originally discovered in skeletal muscle sarcoplasmic reticulum (Ostwald & MacLennan, 1974). Subsequently, it was established that calreticulin is common to both muscle sarcoplasmic reticulum and non-muscle endoplasmic reticulum (Opas *et al.*, 1988; Fliegel *et al.*, 1989*b*).

The localization of calreticulin to reticular membranes has been demonstrated using a variety of biochemical and immunological techniques (Michalak *et al.*, 1980; Lewis *et al.*, 1985, 1986; Koch & Macer, 1988; Opas *et al.*, 1988, 1991; Treves *et al.*, 1990; Milner *et al.* 1991; Michalak *et al.*, 1991; Tharin *et al.*, 1992). First, the protein has been shown to be present in isolated vesicles from the endoplasmic reticulum and sarcoplasmic reticulum (Ostwald & MacLennan, 1974; Michalak *et al.*, 1980, 1991; Lewis *et al.*, 1985; Fliegel *et al.*, 1989*b*; Milner *et al.*, 1991). Calreticulin can be extracted from the lumen of these membrane vesicles by carbonate extraction or by treatment of the vesicles with a low concentration of detergent, indicating that it is a peripheral membrane protein (Ostwald & MacLennan, 1974; Michalak *et al.*, 1980, 1991). That calreticulin is localized to the lumen of these membranes is further supported by molecular cloning of the protein (Fliegel *et al.*, 1989*a*; Smith & Koch, 1989; McCauliffe *et al.*, 1990*a*). These studies indicate that (i) calreticulin terminates with the amino acid sequence KDEL, shown to be responsible for the retention of proteins resident in the endoplasmic reticulum (Pelham, 1989) and (ii) that it is synthesized with an *N*-terminal signal sequence (Fliegel *et al.*, 1989*a*; Rokeach *et al.*, 1991*a*).

Extensive immunocytochemical studies in a variety of different cells confirmed that calreticulin is localized to the endoplasmic reticulum membranes in non-muscle cells (Opas *et al.*, 1988, 1991; Fliegel *et al.*, 1989*b*; Milner *et al.*, 1991; Michalak *et al.*, 1991; Tharin *et al.*, 1992). They have also shown that calreticulin is localized to the reticular membranes of uterine smooth muscle, rat vas deferens smooth muscle and cardiac muscle (Fliegel *et al.*, 1989*b*; Milner *et al.*, 1991; J. Meldolesi, personal communication). Fig. 1(*a*) shows the typical distribution of calreticulin detected with anti-calreticulin antibodies in a well-spread fibroblast. In quadruple localization studies in the same cells, we sequentially visualized calreticulin, RNA-containing organelles, the endoplasmic reticulum, and all intracellular membranes (Michalak *et al.*, 1991; Opas *et al.*, 1991). These results suggest that calreticulin may be confined to, or enriched in, the rough endoplasmic reticulum (Lewis *et al.*, 1985; Opas *et al.*, 1991; Peter *et al.*, 1992). In the same study it was demonstrated that in addition to its endoplasmic reticulum localization, in some cell types calreticulin can also be detected in the nuclear envelope. This is not particularly surprising as the endoplasmic reticulum is continuous with the outer nuclear membrane. However, it remains unclear why this pattern of staining is not seen universally.

Immunolocalization studies have also shown that calreticulin and/or calreticulin-like antigen(s) are present in the nucleus of some cells (Opas *et al.*, 1988, 1991). In proliferating rat L6 muscle cells (myoblasts) calreticulin is evenly and abundantly distributed throughout the endoplasmic reticulum. However, strong intranuclear staining is also observed, localized to the nucleoli. An example of this is shown in Fig. 1(*b*). Interestingly when myoblast fusion was inhibited with either a high serum concentration, or transforming growth factor-β, or 12-*O*-tetradecanoylphorbol 13-acetate, the intranuclear staining disappeared whilst the endoplasmic reticulum staining of calreticulin remained unchanged. In contrast, in differentiated myotubes both intracellular and intranuclear immunoreactivity with anti-calreticulin antibodies are abolished. The reason for this observed localization of calreticulin to 'nucleoli' structures is not clear at present. The primary amino acid sequence of the protein, however, does contain a putative nuclear localization signal (see Fig. 3).

Recently it has been shown that calreticulin is abundant in

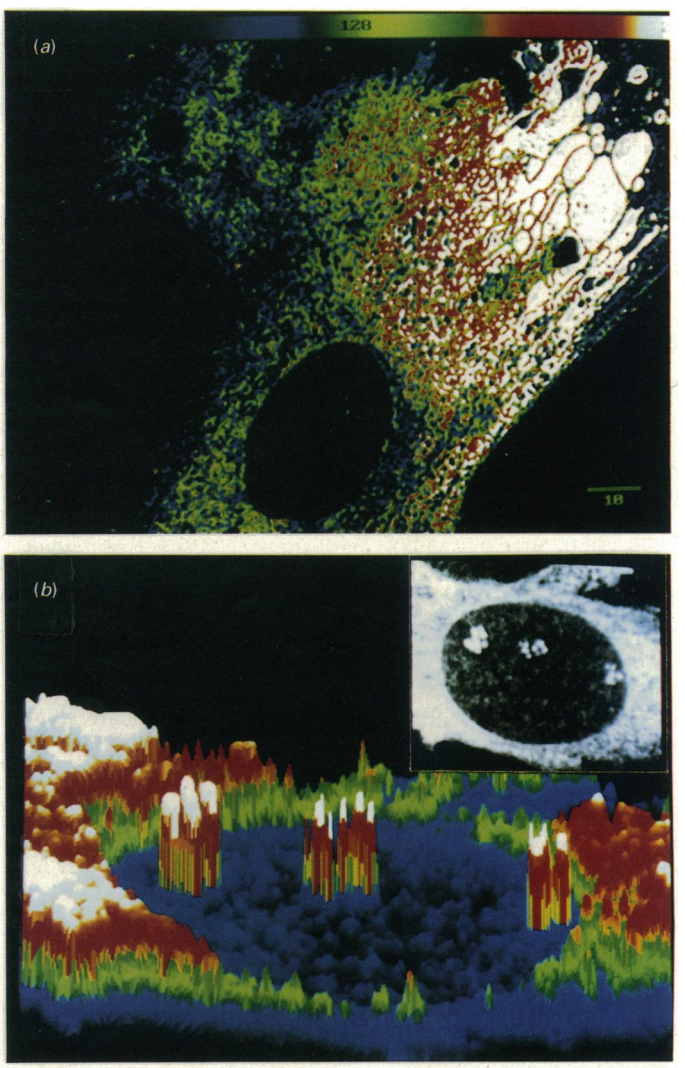

Fig. 1. Intracellular localization of calreticulin

(*a*) Confocal scanning laser microscopy of a pancreatic fibroblast in culture after immunolabelling with specific antibodies against calreticulin. The pattern of calreticulin distribution in this cell closely reflects distribution of the endoplasmic reticulum. The colour bar shows the colour attributes for grey levels 0–255; black to blue corresponds to the non-specific background labelling. (*b*) The intranuclear localization of calreticulin-like antigen(s) in an L6 myoblast, a colour-coded three-dimensional rendition of intensity distribution in a confocal optical section through the nucleus. Optical sectioning of the nuclei reveals that the calreticulin-like antigen(s) localize to discrete foci within nucleoli.

proliferating skeletal muscle myoblasts, but is down-regulated after cellular fusion and the formation of myotubes, suggesting that it might be important during active proliferation and/or protein synthesis (Opas *et al.*, 1991; Michalak *et al.*, 1991). This is further supported by the studies of Gersten *et al.* (1991) who identified calreticulin in B16 melanoma cells as a 50 kDa protein which they referred to as B50. The expression of calreticulin (B50) in these cells appears to be associated with their proliferating activity and may even participate in the host's response to the tumour (Gersten *et al.*, 1989). This result indicates that calreticulin may play an important role during cellular proliferation.

CALRETICULIN–A Ca^{2+} STORAGE PROTEIN OF THE ENDOPLASMIC RETICULUM

Calsequestrin is the major Ca^{2+}-binding (storage) protein in the lumen of muscle sarcoplasmic reticulum (MacLennan *et al.*, 1983; Cala *et al.*, 1990; Milner *et al.*, 1992). It is localized in the junctional sarcoplasmic reticulum (Meissner, 1975; Franzini-Armstrong *et al.*, 1987), where it has been proposed to modulate Ca^{2+}-release processes via the ryanodine receptor/Ca^{2+}-release channel (Ikemoto *et al.*, 1989). The movement of Ca^{2+} ions to and from the non-muscle endoplasmic reticulum is regulated by a group of proteins that are known to be analogous to the proteins of striated and smooth muscle sarcoplasmic reticulum (Walz & Baumann, 1989; Milner *et al.*, 1992). Therefore attempts have been made to identify a non-muscle analogue of calsequestrin. Although several reports have indicated that calsequestrin itself can be detected in non-muscle tissues (Damiani *et al.*, 1988, 1989; Volpe *et al.*, 1988), other reports have contradicted these observations (Scott *et al.*, 1988; Van *et al.*, 1989; Treves *et al.*, 1990; Krause *et al.*, 1990; Milner *et al.*, 1991; Michalak *et al.*, 1991; Opas *et al.*, 1991).

These discrepancies can be explained by the fact that some antibodies raised against calsequestrin cross-react with calreticulin (Volpe *et al.*, 1988; Damiani *et al.*, 1988; Krause *et al.*, 1990; Treves *et al.*, 1990). This cross-reactivity is thought to occur because the two proteins have similar acidic *C*-terminal regions (Fliegel *et al.*, 1987, 1989*a*; Scott *et al.*, 1988) that are probably involved in the high-capacity Ca^{2+} binding observed in both molecules (Ohnishi & Reithmeier, 1987; Baksh & Michalak, 1991). In addition, calreticulin and calsequestrin share several '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 (MacLennan *et al.*, 1983; Fliegel *et al.*, 1989*b*; Krause *et al.*, 1990). Despite these apparent similarities, the amino acid sequences of calreticulin and calsequestrin indicate that they are different proteins (Fliegel *et al.*, 1987, 1989*a*; Scott *et al.*, 1988; Milner *et al.*, 1991) and in fact structurally they have very little in common (their overall identity is less than 10%). Antibodies produced in our laboratory against calreticulin do not cross-react with calsequestrin and, conversely, the antibodies raised against calsequestrin do not recognize calreticulin (Fliegel *et al.*, 1989*b*; Milner *et al.*, 1991) allowing us to demonstrate that calreticulin, and not calsequestrin, is a major Ca^{2+}-binding protein of non-muscle and smooth muscle endoplasmic and sarcoplasmic reticulum (Milner *et al.*, 1991).

In sarcoplasmic reticulum membranes the major Ca^{2+}-binding (storage) protein is calsequestrin (MacLennan *et al.*, 1983; Cala *et al.*, 1990). In contrast, the non-muscle endoplasmic reticulum appears to contain a group of Ca^{2+}-binding proteins, all of which may contribute to the overall Ca^{2+} storage capacity of the lumen of these membranes (Koch *et al.*, 1986, 1989; Macer & Koch, 1988; Van *et al.*, 1989; Nigam & Towers, 1990; Milner *et al.*, 1991; Michalak *et al.*, 1991). This group includes protein disulphide isomerase, immunoglobulin heavy-chain binding protein (BiP; Grp78), and endoplasmin (Grp94) as well as calreticulin. These proteins have been referred to as the reticuloplasmins (Koch, 1987). It has been reported that together these four proteins account for the major proportion of the Ca^{2+}-binding capacity of the endoplasmic reticulum (Macer & Koch, 1988), which is calculated to be approx. 300 nmol of Ca^{2+}/mg of protein (Macer & Koch, 1988).

That Ca^{2+} binds to protein disulphide isomerase, BiP, calreticulin and endoplasmin is not surprising considering that they all contain similar clusters of acidic residues at their *C*-terminals (Fliegel *et al.*, 1989*c* and references therein). These acidic residues are known to be involved in high-capacity Ca^{2+} binding to calreticulin (Baksh & Michalak, 1991) and calsequestrin (Ohnishi & Reithmeier, 1987), and by analogy they are likely to be involved in Ca^{2+} binding to protein disulphide isomerase, BiP and endoplasmin. The relative amount and acidity of each protein will determine its contribution to the Ca^{2+} storage capacity of the endoplasmic reticulum. Calreticulin is known to have a particularly high Ca^{2+} binding capacity (approx 200 nmol of Ca^{2+}/mg of protein) (MacLennan *et al.*, 1972; Treves *et al.*, 1990; Michalak *et al.*, 1991; Baksh & Michalak, 1991). Endoplasmin also binds large amounts of Ca^{2+} (approx. 280 nmol/mg of protein) (Macer & Koch, 1988). When these binding values are expressed as mol of Ca^{2+} bound per mol of protein, it appears that calreticulin binds up to about 25 mol of Ca^{2+}/mol, whereas endoplasmin binds up to about 10 mol/mol. Both proteins are therefore likely to contribute significantly to Ca^{2+} storage within the endoplasmic reticulum. Two additional Ca^{2+}-binding proteins (CaBP1 and CaBP2) have been found by Van *et al.* (1989) in rat liver microsomal vesicles, but their identity and contribution to the Ca^{2+}-storage capacity of the endoplasmic reticulum membrane has not yet been established. The Ca^{2+} binding properties of BiP and PDI have also not yet been established. However, these proteins have been proposed to play an important role in protein translocation, folding and synthesis (Gething & Sambrook, 1992). Given the Ca^{2+}-binding capabilities of calreticulin, and the fact that it shares several 'diagnostic' biochemical properties with calsequestrin, it was proposed that calreticulin is a major Ca^{2+}-binding (storage) protein in the lumen of the endoplasmic reticulum (Milner *et al.*, 1991).

THE DISTRIBUTION OF CALRETICULIN

In early studies, a radioimmunoassay was used to determine the amount of calreticulin in different bovine tissues and it was found to be present in all tissues tested except for erythrocytes (Khanna & Waisman, 1986). Calreticulin was found in particularly high concentration (200–500 μg/g of tissue) in pancreas, liver and testis. In contrast, kidney, spleen, adrenals and parathyroid had only moderate amounts (100 μg/g of tissue) and the cerebral cortex and muscle tissues had relatively low amounts (approx. 20 μg/g of tissue). More recently, the development of a simple $(NH_4)_2SO_4$ precipitation procedure for the isolation of native and recombinant calreticulin (Fliegel *et al.*, 1989*b*; Krause *et al.*, 1990; Baksh & Michalak, 1991; Rokeach *et al.*, 1991*b*; Baksh *et al.*, 1992) has provided a convenient tool for the identification of a number of different calreticulins (Collins *et al.*, 1989; Damiani *et al.*, 1989; Krause *et al.*, 1990; Treves *et al.*, 1990; Milner *et al.*, 1991; Michalak *et al.*, 1991; Baksh & Michalak, 1991). Fig. 2 shows the *N*-terminal amino acid sequences obtained for calreticulins isolated from a variety of different tissues and species.

In addition to its wide tissue distribution, calreticulin has been

Protein source		Amino acid sequence	Reference
		10 20 30	
Fast-twitch skeletal muscle[a, b]	Rabbit	EPVVYFKEQF LDGDGWTDRW IESKHKSDF	[1]
Slow-twitch skeletal muscle[b]	Rabbit	EPVVYFQFQF LDGDGWTDRW IESKHKSDF	[2, 3]
Brain[a]	Rabbit	EPVVYFKEQF LDGDGWTERG IESKHKSDF	[3, 4]
Uterus[a]	Rabbit	EPVVYFKEQF LDGDGWTDRW IESKHKSDF	[3]
Liver[a]	Rabbit	EPVVYFKEQF LDGDGWTDRW	[3–5]
Lung[a]	Rabbit	EPVVYFKxxx xxGDGWTERW IESKHKSDF	[6]
Uterus[a]	Porcine	EPTIYFKEQF LDGDG	[3]
Liver[a]	Bovine	EPAIYFKEQF LDG	[3]
Melanoma cells (B50)[a]	Mouse	xxAIYFKEQF LNGNA	[7]
Plasmacytoma cells (calreticulin/CRP55)[a, b]	Mouse	DPAIYFKEQF LNGNAWTNRW VESKHKSDF	[8]
Pancreas[a]	Dog	EPAIYFKEQF LDGxGFTDxR IKEK	[9]
Brain[a]	Dog	EPAIYFKEQF LNGNAxTNRx VES	[10]
Liver (calregulin)[a]	Chicken	EPAQFFKEEK LNGNA	[5]
Chick embryo 53 kDa protein[a]	Chicken	TPAQFFREEF LDGDDWTQRD V	[11]
Liver (CaBP3)[a]	Rat	DPAIYFKEQF LNGNAATNR	[12]
Calreticulin[b]	Rat	DPAIYFKEQF LDGDAWTNRW VESKHKSDF	[13]
Fibroblast (p425)[a]	Rat	xPTxYFxEQF LNGNxRA	[14]
Liver[a]	Rat	DPAIYPKEQF LD	[4]
Neurons (p407 'memory molecule')[a]	Aplysia	xPTIYFKEEF GNDxAE	[14]
Wil-2 cell line (Ro/SS-A/calreticulin)[a, b]	Human	EPAIYFKEEF LDGDGWTSRW IESKHKSDF	[15]
B-Lymphocytes (calreticulin)[b]	Human	EPAVYFKEQF LDGDGWTSRW IESKHKSDF	[16]
HL-60 (calreticulin)[a]	Human	EPAIYFKEEF LDGDG	[17]

Fig. 2. *N*-terminal amino acid sequence of different forms of calreticulin

Amino acid sequences are taken from: 1, Fliegel *et al.* (1989*a*); 2, Fliegel & Michalak (1991); 3, Milner *et al.* (1991); 4, Treves *et al.* (1991); 5, Khanna *et al.* (1987); 6, Guann *et al.* (1991); 7, Gersten *et al.* (1991); 8, Smith & Koch (1989); 9, Michalak *et al.* (1991); 10, Collins *et al.* (1989); 11, Bassuk & Berg (1991); 12, Van *et al.* (1989); 13, Murthy *et al.* (1990); 14, Kennedy *et al.* (1988); 15, McCauliffe *et al.* (1990*a*); 16, Rokeach *et al.* (1991*a*); 17, Krause *et al.* (1990). x, residue not determined; [a] data derived from protein sequencing; [b] amino acid sequences deduced from cDNA sequence. Identical residues and conserved substitutions are in black; significantly different residues are in red.

shown to be present in a number of extremely diverse species (see Fig. 2; Lewis *et al.*, 1985; Kennedy *et al.*, 1988; Fliegel *et al.*, 1989*a*,*b*; Smith & Koch, 1989; Collins *et al.*, 1989; McCauliffe *et al.*, 1990*a*; Murthy *et al.*, 1990; Krause *et al.*, 1990; Treves *et al.*, 1990; Milner *et al.*, 1991; Rokeach *et al.*, 1991*a*; Gersten *et al.*, 1991; Bassuk & Berg, 1991; Perrin *et al.*, 1991; Michalak *et al.*, 1991; Opas *et al.*, 1991; Tharin *et al.*, 1992). Importantly, calreticulin has been identified, by *N*-terminal amino acid sequence analysis, molecular cloning and immunological analysis, in all eukaryotic cells so far studied. This includes the recent identification of calreticulin in plant tissues (Allen & Tiwari, 1991; R. E. Milner, M. Opas & M. Michalak, unpublished work).

Independently of these studies, other forms of calreticulin have also been identified. For example, the *Aplysia* p407 protein, the expression of which is modulated during long-term sensitization (Kennedy *et al.*, 1988), was found to have an *N*-terminal amino acid sequence similar to that of calreticulin (Fig. 2). The amino acid sequence of *Aplysia* p407 protein has now been deduced from its cDNA and indicates that it is the *Aplysia* homologue of calreticulin (T. E. Kennedy & E. R. Kandel, personal communication). The acquisition of long-term memory appears to depend to some extent on the induction of protein synthesis (Kennedy *et al.*, 1992). As yet there is no indication of the role that calreticulin might play in these processes, but its up-regulation suggests that it may be of fundamental importance.

Recent Northern blot analysis of the distribution of calreticulin mRNA has further supported our conclusion that calreticulin is a wide-spread and abundant cellular protein. mRNA encoding calreticulin (approx. 1.9 kb) has been identified in a variety of different tissues including rabbit fast-twitch and slow-twitch skeletal muscle, cardiac muscle, smooth muscle, liver, kidney, brain, pancreas, mouse splenic cells, human peripheral blood leukocytes, human Wil-2 cells, a human hybridoma cell line, a Jurkat T-cell line, Raji cells, HeLa cells, African Green Monkey cells, mouse pancreas and tobacco NTαPh cells (Fliegel *et al.*, 1989*a*; McCauliffe *et al.*, 1990*a*; Fliegel & Michalak, 1991; Milner *et al.*, 1991; Michalak *et al.*, 1991; Rokeach *et al.*, 1991*a*). Under conditions of relatively high stringency, we have detected hybridization of calreticulin cDNA to another, larger species of mRNA (3.75 kb in length) (Fliegel *et al.*, 1989*a*). The precise identity of this mRNA is not clear at present. It may, however, correspond to either an unspliced mRNA species or an mRNA species encoding a different protein which shares some sequence similarities with calreticulin.

THE AMINO ACID SEQUENCE OF CALRETICULIN

Fig. 3 shows the amino acid sequence of rabbit skeletal muscle calreticulin, which was deduced from the nucleotide sequence of cDNA encoding this protein (Fliegel *et al.*, 1989*a*). The localization of calreticulin to the lumen of endoplasmic/sarcoplasmic reticulum membranes (Michalak *et al.*, 1980) suggested that the protein might require a signal sequence. Structural analysis of the deduced amino acid sequence of calreticulin indicates that the protein has an hydrophobic *N*-terminal signal sequence (Fig. 3) (Fliegel *et al.*, 1989*a*; Smith & Koch, 1989; McCauliffe *et al.*, 1990*a*), although it is somewhat atypical in that it lacks a basic residue near its *N*-terminus (von Heijne, 1985). The presence of a signal sequence was confirmed by *in vitro*

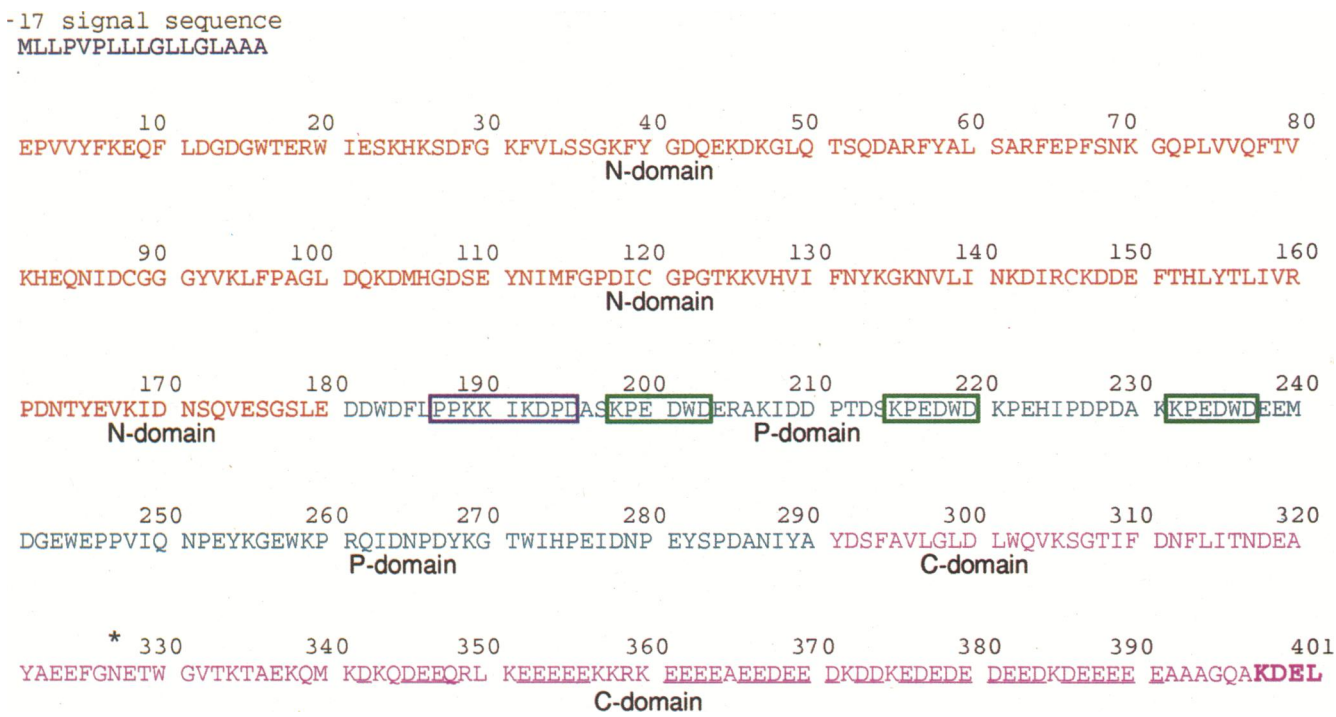

Fig. 3. Amino acid sequence of rabbit skeletal muscle calreticulin

The amino acid sequence of calreticulin is based on the amino acid sequence deduced from the nucleotide sequence of a cDNA clone encoding the skeletal muscle protein (Fliegel *et al.*, 1989*a*). N-domain, P-domain and C-domain have been presented in red, blue and purple; acidic residues in the C-domain are underlined; the green boxes contain KPEDWD repeats; and the purple box a putative nuclear localization signal (NLS). The asterisk indicates a possible glycosylation site.

translation of mRNA encoding calreticulin (Fliegel *et al.*, 1989*a*; Rokeach *et al.*, 1991*a*).

Mature calreticulin (M_r 46567) contains 109 acidic and 52 basic amino acids (Fig. 3). The sequence contains no long hydrophobic segments capable of spanning the membrane bilayer (Fliegel *et al.*, 1989*a*; Smith & Koch, 1989; McCauliffe *et al.*, 1990*a*), confirming earlier indications that mature calreticulin is a peripheral membrane protein (Ostwald & MacLennan, 1974; Michalak *et al.*, 1980, 1991). Structural predictions suggest that the first half of the molecule forms a globular domain of eight anti-parallel β-strands with a helix–turn–helix motif at the extreme *N*-terminus. The next one-third of the sequence is proline-rich, and can be subdivided into a charged region which contains a 17-amino-acid repeat (PxxIxDPDAxKPEDWDE), followed by a proline-, serine- and threonine-rich segment. This is then followed by the *C*-terminus, which contains 37 acidic residues.

Searches for different functional motifs or consensus sequences within the amino acid sequence of calreticulin have revealed several interesting possibilities. As mentioned previously, one potential glycosylation site is found (residue 326). However, thus far only bovine (Waisman *et al.*, 1985) and rat (Van *et al.*, 1989) liver proteins appear to be glycosylated. The protein has putative recognition sequences for phosphorylation by protein kinase C (clustered at the *N*-terminal domain of the protein; residues 17–19, 36–38, 61–63, 68–70, 79–81 and 124–126), casein kinase II (residues 51–54, 172–175, 178–181, 196–200, 204–208, 307–311 and 316–319) and tyrosine kinase (residues 261–268). However, we have failed to show any phosphorylation of either native or recombinant calreticulin by protein kinase C or tyrosine kinase (C. Shemanko, R. E. Milner & M. Michalak, unpublished work). Calreticulin also appears to have a sequence with marked similarity to the active site of protein kinase C (residues 215–224) (Hanks *et al.*, 1988), but Waisman *et al.* (1985) were unable to detect any kinase activity associated with purified bovine liver calreticulin. Two lysosome targeting signals are also found in calreticulin's amino acid sequence (residues 42–48 and 347–353) (Dice, 1990). McCauliffe *et al.* (1990*a*) noted that calreticulin contains several regions rich in proline, glutamic acid, serine and/or threonine residues. These regions, termed 'PEST' (Rogers *et al.*, 1986), are thought to make a protein susceptible to rapid intracellular degradation. One of the more interesting features of the calreticulin sequence is the presence of a nuclear localization signal (PPKKIKPDP; residues 187–195) (Fig. 3) (McCauliffe *et al.*, 1990*a*; Opas *et al.*, 1991). This may be particularly relevant given the recent detection of calreticulin in the nucleus (Opas *et al.*, 1991). Most importantly, the endoplasmic reticulum retention sequence KDEL (Pelham, 1989) is found in calreticulin (Fliegel *et al.*, 1989*a*; Smith & Koch, 1989; McCauliffe *et al.*, 1990*a*). As yet, none of the motifs described in calreticulin have been proven to have functional significance.

Full length amino acid sequences have now been deduced for numerous calreticulins (rat, human, mouse, *Drosophila*), from the nucleotide sequences of cDNA (Smith & Koch, 1989; McCauliffe *et al.*, 1990*a*,*b*; Murthy *et al.*, 1990; Rokeach *et al.*, 1991*a*). These sequences confirm the suggestion made earlier, on the basis of *N*-terminal amino acid sequence analysis, that calreticulin is a highly conserved protein. Fig. 4 shows the complete amino acid sequences of rabbit skeletal muscle, mouse plasmacytoma cell, rat brain and human Wil-2 B-cell line calreticulin, as well as partial amino acid sequences of *Drosophila* calreticulin and of the *Onchocerca volvulus* Ral-1 antigen. There is over 90% amino acid sequence identity between rabbit, mouse, human and rat calreticulins (Fig. 4). In addition, although the

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                10        20        30        40        50        60        70        80
Rabbit     EPVVYFKEQF LDGDGWTERW IESKHKSDFG KFVLSSGKFY GDQEKDKGLQ TSQDARFYAL SARFEPFSNK GQPLVVQFTV
Human      EPVVYFKEQF LDGDGWTSRW IESKHKSDFG KFVLSSGKFY GDEEKDKGLQ TSQDARFYAL SASFE-FSNK GQPTVVQFTV
Mouse      DPVIYFKEQF LDGDAWTNRW VESKHKSDFG KFVLSSGKFY GDLEKDKGLQ TSQDARFYAL SAKFE-FSNK GQPLVVQFTV
Rat        DPAIYFKEQF LDGDAWTNRW VESKHKSDFG KFVLSSGKFY GDQEKDKGLQ TSQDARFYAL SARFEPFSNK GQTLVVQFTV
Drosphila                                  partial sequence                              LVVQFSV
RAL-1                  incomplete NH2-end          GDAVKDKGLK TTQDAKFYSI CAKFDKSSNK GQKSVVIFTS

                90        100       110       120       130       140       150       160
Rabbit     KHEQNIDCGG GYVKLFPAGL DQKDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR
Human      KHEQNIDCGG GYVKLFPNSL DQTDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR
Mouse      KHEQNIDCGG GYVKLFPSGL DQKDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR
Rat        KHEQNIDCGG GYVKLFPDGL DQKDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR
Drosphila  KHEQNIDC-A GYVKLFDCGL DQKDMHG       partial sequence
RAL-1      KHEQNDDCGG GYVKLMASDV NLEDSHGETP YHIMFGPDIC GPGTKKVHVI FHYKDRNHMI KKDIRCKDDV FTHIYTLIVN

               170       180       190       200       210       220       230       240
Rabbit     PDNTYEVKID NSQVESGSLE DDWDFLPPKK IKDPDASKPE DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM
Human      PDNTYEVKID NSQVESGSLE DDWDFLPPKK IKDPDASKPE DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM
Mouse      PDNTYEVKID NSQVESGSLE DDWDFLPPKK IKDPDAAKPE DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM
Rat        PDNTYEVKID NSQVESGSLE DDWDFLPPKK IKDPDAAKPE DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM
Drosphila     YELLID NEKVESGNLE DDWDFLAPKK IKDPTATKPE DWD     partial          sequence
RAL-1      SDNTYFVQID GFKAESGELE ADWDFLPPKK IKDPDAKKPE DWDEREFIDD EDDKKPEDWD KPEHIPDPDA KKPEDWDDEM

               250       260       270       280       290       300       310       320
Rabbit     DGEWEPPVIQ NPEYKGEWKP RQIDNPDYKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD LWQVLSGTIF DNFLITNDEA
Human      DGEWEPPVIQ NPEYKGEWKP PQIDNPDYKG TWIHPEIDNP EYSPDPSIYA YDNFGVLGLD LWQVKSGTIF DNFLITNDEA
Mouse      DGEWEPPVIQ NPEYKGEWKP PQIDNPDWKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD LWQVKSGTIF DNFLITNDEA
Rat        DGEWEPPVIQ NPEYKGEWKP RQIDNPDYKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD LWQVKSGTIF DNFLITNDEA
RAL-1      DGEWEPPMVD NPEYKGEWKP KQKKNPAWKG KWIHPEIEIP DYTPDDNLYV YDDIGAIGFD LWQVLSGTIF DDVIVTDSVE

               330       340       350       360       370       380       390       401
Rabbit     YAEEFGNETW GVTKTAEKQM KDKQDEEQRL KEEEEEKKRK EEEEAEEDEE DKDDKEDEDE DEEDKDEEEE EAAAGQAKDEL
Human      YAEEFGNETW GVTKTAEKQM KDKQDEEQRL KEEEEEKKRK EEEEAEDKED D-EEKDEDEE DEEDKEEDEE EDVPGQAKDEL
Mouse      YAEEFGNETW GVTKTAEKQM KDKQDEEQRL KEEEEEKKRK EEEEAEDKED D-EDRDEDED EEDEKEEDEE ES-PGQAKDEL
Rat        YAEEFGNETW GVTKAAEKQM KDKQDEEQRL KEEEEDKKRK EEEEAEDKED E-DDRDEDED DEEDEKEEDE EDATGQAKDEL
RAL-1      EAKKFGEKTL KKIKREGKQ- KD----GQKT ------KKRK -EKEKNEKIK EKMKKRKRAN R-KKKK
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Fig. 4. Amino acid sequence similarities of different calreticulins

Comparison of rabbit, human, mouse, rat, *Drosophila* calreticulins and the RAL-1 *Onchocercal* 'homologue' is shown. Black represents perfect matches and conserved residues; red shows different residues; (–) represents a gap. The amino acid sequences were deduced from the nucleotide sequences of different cDNAs encoding the proteins (Fliegel *et al.*, 1989*a*; Smith & Koch, 1989; McCauliffe *et al.*, 1990*a*,*b*; Murthy *et al.*, 1990; Unnasch *et al.*, 1988).

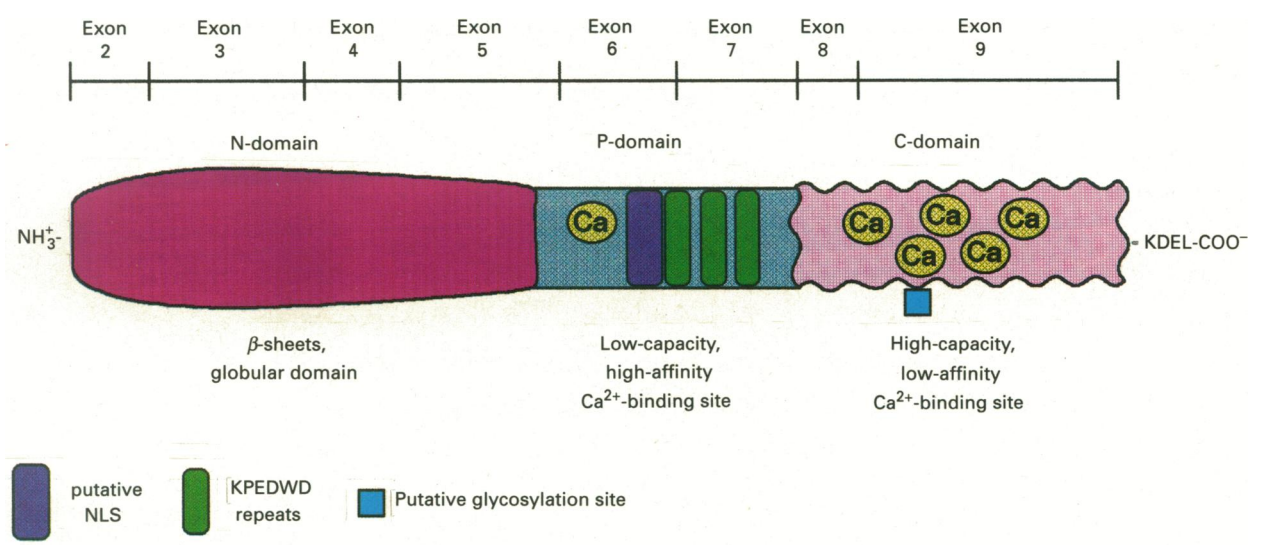

Fig. 5. Putative model of calreticulin domains

This is a schematic illustration representing the various domains of calreticulin including the localization of KPEDWD repeats, a putative nuclear localization signal (NLS), a putative glycosylation site and Ca^{2+}-binding sites. The exon–intron boundaries were identified by McCauliffe *et al.* (1992). Exon 1, encoding the signal sequence of calreticulin, is not shown.

Drosophila cDNA clone has only been partially sequenced (McCauliffe *et al.*, 1990*b*; Fig. 3) it shows over 80% sequence similarity with other calreticulins, confirming that *Drosophila* has a gene which encodes calreticulin. The rabbit calreticulin sequence is also about 60% identical to the partially sequenced *Onchocerca* Ral-1 molecule (Unnasch *et al.*, 1988; Fig. 4). Importantly, many of the *Drosophila* residues that differ from the human, mouse, rat and rabbit sequences are identical to the *Onchocerca* residues, indicating phylogenetic conservation of this protein (McCauliffe *et al.*, 1990*b*). The sequence similarities between the mammalian calreticulins, the *Drosophila* calreticulin and the *Onchocerca* Ral-1 antigen are striking, especially when it is considered that mammals diverged phylogenetically approximately 75 million years ago and *Drosophila* and *Onchocerca* diverged over 350 million years ago (Barrett *et al.*, 1986). As discussed in an earlier section, 'calreticulin' has also been identified in plant cells, but determination of the degree of similarity between plant 'calreticulin' and the previously identified calreticulins awaits the cloning and sequencing of its cDNA.

It is interesting to note that in spite of the slight variations in the amino acid sequences of the different calreticulins, there are several areas where they are invariant (Fig. 4). These include a sequence predicted to form highly structured antiparallel β-strands (residues 130–148), and a proline-rich region (residues 232–254). The areas that are most conserved might well be areas of greatest structural and functional importance. The area of least homology, particularly in the *Onchocerca* protein, is at the *C*-terminus. This end of the *Onchocerca* protein is highly charged, as it is in the other calreticulins, but it is positively rather than negatively charged (Unnasch *et al.*, 1988; McCauliffe *et al.*, 1990*b*). Since the *C*-terminal, negatively charged region of calreticulin is involved in the high-capacity and low-affinity binding of Ca^{2+} to the protein (Baksh & Michalak, 1991), the *Onchocerca* protein would not be expected to show this type of binding. However, the high-affinity and low-capacity Ca^{2+}-binding site that is localized to the proline-rich domain in calreticulin, is found in the *Onchocerca* protein (Unnasch *et al.*, 1988; McCauliffe *et al.*, 1990*b*; Baksh & Michalak, 1991). Another difference between the *Onchocerca* protein and calreticulin is that the former does not terminate with the KDEL endoplasmic reticulum retention signal (Unnasch *et al.*, 1988), suggesting that, in contrast to calreticulin, the *Onchocerca* protein may not be retained in the lumen of the endoplasmic reticulum. The *Onchocerca* calreticulin will provide an important tool to study the function of the different putative domains of calreticulin.

Wada *et al.* (1991), whilst studying membrane proteins involved in the translocation of nascent chains across the endoplasmic reticulum, cloned an integral membrane protein of the endoplasmic reticulum which shows marked similarity to calreticulin in its amino acid sequence. The *C*-terminal 249 amino acids of calreticulin, which contain its Ca^{2+} binding domains (Baksh & Michalak, 1991), appear to form a part of this larger 90 kDa transmembrane polypeptide which has been termed calnexin (Wada *et al.*, 1991). The high similarity between the two proteins is confined to the central and *C*-terminal regions of calreticulin (Wada *et al.*, 1991) which have previously been designated as the P- and C-domain, respectively (Fig. 5) (Baksh & Michalak, 1991). In calnexin the two domains of 'calreticulin' are separated by a predicted transmembrane sequence unique to calnexin. Calreticulin does not contain any amino acid sequences predicted to form transmembrane segments (Fliegel *et al.*, 1989*a*). It has been postulated that calnexin might form part of an oligomeric complex involved in the translocation of nascent chains across the endoplasmic reticulum membrane (Wada *et al.*, 1991).

A search for sequence homology between calreticulin and other proteins has led to the determination of amino acid identities (similarities) between the *C*-terminal region of calreticulin and a number of other proteins, including protein disulphide isomerase, BiP (Grp78), Grp94 (endoplasmin) and some heat-shock proteins (Fliegel *et al.*, 1989*c*). All of these proteins, including calreticulin, terminate with the endoplasmic reticulum retention signal KDEL, preceded by clusters of acidic residues. It has been proposed that Ca^{2+} might be involved in the regulation of the interaction of KDEL proteins with the KDEL receptor (Kelly, 1990; Vaux *et al.*, 1990; Lewis & Pelham, 1990). Ca^{2+} bound to the *C*-terminus of these proteins may, therefore, be involved in these interactions. This suggestion is supported by the observation that the addition of the KDEL sequence to proteins without upstream acidic residues leads to only partial retention of these molecules within the lumen of the endoplasmic reticulum (Zagouras & Rose, 1989). The *C*-terminus of

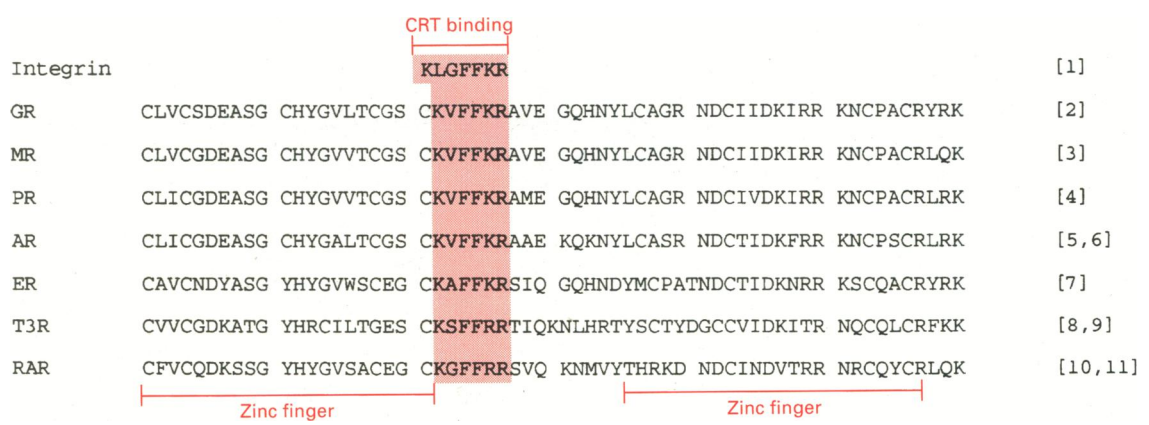

Fig. 6. The amino acid sequence of a calreticulin binding peptide and the DNA-binding domain of steroid hormones

GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; AR, androgen receptor; OR, oestrogen receptor; T3R, thyroid hormone receptor; RAR, retinoic acid receptor; CRT, calreticulin. Partial amino acid sequences of different receptors are taken from: 1, Rojiani *et al.* (1991); 2, Hollenberg *et al.* (1985); 3, Arriza *et al.* (1987); 4, Misrahi *et al.* (1987); 5, Chang *et al.* (1988); 6, Lubahn *et al.* (1988); 7, Walter *et al.* (1985); 8, Sap *et al.* (1986); 9, Weinberger *et al.* (1986); 10, Giguere *et al.* (1987); 11, Petkovitch *et al.* (1988).

calreticulin and of other KDEL proteins may, therefore, have a dual function: (i) Ca^{2+} storage and (ii) control of the retention of the protein in the lumen of the endoplasmic reticulum.

Recently the calreticulin gene was isolated and analysis of the promoter region revealed that it contains a number of potential regulatory sites that are also found in the genes for other KDEL proteins [e.g. BiP (Grp78), Grp94 (endoplasmin), and PDI protein disulphide isomerase] (McCauliffe *et al.*, 1992). These sites include multiple Sp1 and CCAAT consensus sequences, an AP-2 recognition sequence (absent from protein disulphide isomerase), and multiple G+C-rich areas. These data suggest that calreticulin, BiP, Grp94 and protein disulphide isomerase might have similar transcriptional regulation. BiP and Grp94 show extensive sequence similarity with the stress-induced proteins Hsp70 and Hsp90/83 (Sorger & Pelham, 1987; Nicholson *et al.*, 1990). This entire family of proteins, including protein disulphide isomerase, is thought to play some role in protein transport, folding, and assembly (Sorger & Pelham, 1987; Rothman, 1989; Gething & Sambrook, 1992). The promoter region of the calreticulin gene contains AP-2 (Imagawa *et al.*, 1987) and H4TF-1 (Dailey *et al.*, 1986) recognition sequences, which are typically found in genes that are active during cellular proliferation. Their presence in the calreticulin gene is consistent with the finding that calreticulin is expressed at elevated levels in rapidly dividing cells (Opas *et al.*, 1991; Gersten *et al.*, 1991; McCauliffe *et al.*, 1992).

The calreticulin gene is localized to human chromosome 19, occupies approx. 6 kb of genomic DNA, is not highly polymorphic and exists in a single copy (McCauliffe *et al.*, 1990*a*). The 6 kb human genomic clone isolated by McCauliffe *et al.* (1992) contains nine exons, eight introns, and several hundred base pairs 3′ of the polyadenylation sequence. In total, the introns contribute about 2.4 kb to the gene, but four of the eight are shorter than 100 bp (McCauliffe *et al.*, 1992). The location of the intron–exon boundaries found in the human gene in relation to the amino acid sequence of full length mature calreticulin are shown in Fig. 5.

CALRETICULIN HAS DISTINCT Ca^{2+} BINDING DOMAINS

Analysis of the amino acid sequence of calreticulin indicated that it might be divided into distinct structural domains that may perform different functions (Fliegel *et al.*, 1989*a*; Smith & Koch, 1989; Baksh & Michalak, 1991; Fig. 5). This is supported by the demonstration that the high-affinity and the low-affinity Ca^{2+}-binding sites are localized to different regions in the protein (Baksh & Michalak, 1991).

The *N*-terminal half of the protein (designated the N-domain) is proposed to be a globular domain containing eight antiparallel β-strands. This region leads into a proline-rich sequence (the P-domain) followed by the *C*-terminal quarter of the protein (the C-domain) (Fig. 5). A portion of the P-domain sequence (residues 266–295) contains a number of proline residues spaced every four or five amino acids (Fig. 3). We have suggested that this sequence might contain a repeating, rigid turn structure which separates the globular head of the protein from the acidic tail (Fliegel *et al.*, 1989*a*). The C-domain of calreticulin is acidic; in the last 57 residues, 37 are aspartic or glutamic acid (Fliegel *et al.*, 1989*a*: Smith & Koch, 1989). This domain terminates with the endoplasmic reticulum retention signal, KDEL.

Calreticulin, and the domains of calreticulin, were expressed as recombinant proteins in *Escherichia coli* (Baksh & Michalak, 1991) using a glutathione *S*-transferase fusion protein system developed by Smith & Johnson (1988). This was followed by functional studies aimed at localization of the Ca^{2+}-binding domains in calreticulin (Baksh & Michalak, 1991). The studies demonstrated that the low-affinity, high-capacity Ca^{2+} binding is localized to the *C*-terminal region (C-domain) of the protein, whilst the high-affinity Ca^{2+}-binding site is specifically localized to the P-domain (Baksh & Michalak, 1991) (Fig. 5). In these studies it was noted that both P- and C-domains of the protein contribute to the blue staining of mature calreticulin observed with Stains-All (Baksh & Michalak, 1991).

The localization of the low-affinity and high-capacity Ca^{2+} binding sites to the C-domain of calreticulin is not surprising, since this part of the protein consists of clusters of aspartic and glutamic acid residues (Baksh & Michalak, 1991). Along with calreticulin, several other known Ca^{2+}-binding proteins have clusters of acidic amino acid residues that have been implicated to be involved in their observed Ca^{2+} binding. These include chromogranin A, a Ca^{2+} binding protein found in chromaffin granules in a variety of endocrine and neuronal cells (Simon & Aunis, 1989; Yoo & Albanesi, 1991; Helman *et al.*, 1988) and the ryanodine receptor/Ca^{2+} channel of sarcoplasmic reticulum membranes (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). Similar clusters of acidic residues in the *C*-terminal region of skeletal and cardiac muscle calsequestrin (Fliegel *et al.*, 1987; Scott *et al.*, 1988) have recently been shown to be directly involved in the high-capacity, low-affinity Ca^{2+} binding of this protein (Ohnishi & Reithmeier, 1987). The capacity for low-affinity Ca^{2+} binding is different in calreticulin and calsequestrin (calsequestrin approx. 50 mol/mol of protein versus calreticulin approx. 20–25 mol/mol of protein), despite the fact that the number of acidic residues in both proteins is very similar. However, close examination of the *C*-terminal region of these two proteins shows significant differences in their sequences. In particular, whilst a majority of the last 50 amino acids in both proteins is highly acidic, in the case of the two isoforms of calsequestrin this region consists of long, uninterrupted 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. This difference would probably affect both the folding and the net charge of this region in the two proteins, resulting in the differing capacity for Ca^{2+} binding. The C-domain of calreticulin appears, *in vitro*, to bind Mg^{2+} as well as Ca^{2+} (Baksh & Michalak, 1991). The significance of this observation has yet to be determined. However, it has been suggested that in the endoplasmic reticulum Mg^{2+} may balance charge movement related to Ca^{2+} release (Baumann *et al.*, 1991) and therefore it is possible that, *in vivo*, calreticulin might have some role in binding Mg^{2+} within the lumen of the endoplasmic reticulum.

The high-affinity Ca^{2+}-binding site on calreticulin is located in the P-domain. This domain binds approximately 1 mol of Ca^{2+}/mol of protein with a dissociation constant of 6–11 μM. The best known high-affinity Ca^{2+}-binding proteins are members of the EF-hand family (Kretsinger *et al.*, 1988; Heizmann & Hunziker, 1991) but it is notable that the amino acid sequence of calreticulin does not contain an EF-hand consensus sequence (Fliegel *et al.*, 1989a; Smith & Koch, 1989). The annexins, another widely distributed family of proteins, bind phospholipids and cellular membranes in a Ca^{2+}-dependent manner (Klee, 1988; Burgoyne & Geisow, 1989; Smith *et al.*, 1990; Crumpton & Dedman, 1990; Hazarika *et al.*, 1991*a*,*b*) and have recently been recognized to contain a different type of high-affinity Ca^{2+} binding site. However, annexins also share no homology with calreticulin. Therefore, calreticulin probably contains a novel motif for high-affinity Ca^{2+} binding and may belong to a third, undescribed family of high-affinity Ca^{2+}-binding proteins. The structural features of the P-domain that are responsible for the high-affinity Ca^{2+}-binding remain to be determined. This domain

does contain a relatively high concentration of aspartic acid (24 mol%) and glutamic acid (15 mol%) residues and it is possible that some of these may be involved in the binding.

CALRETICULIN AND AUTOIMMUNITY

On the basis of *N*-terminal amino acid sequence analysis it was recently proposed that the Ro/SS-A antigen might in fact be calreticulin (Liu *et al.*, 1988; Collins *et al.*, 1988). This suggestion was subsequently supported by the isolation and sequencing of a complete cDNA encoding the Ro/SS-A antigen (McCauliffe *et al.*, 1990*a*,*b*). In contrast, however, Rokeach *et al.* (1991*a*) have reported evidence that strongly suggests that calreticulin is not the Ro/SS-A autoantigen. These antigen(s) are of clinical importance since antibodies directed against them are found in the majority of patients with primary Sjögren's syndrome and with systemic lupus erythematosus. Although these findings require clarification, the evidence suggests that calreticulin may share epitopes with the Ro/SS-A autoantigen, or that it may have some secondary role in the pathology of this autoimmune disease. As mentioned previously, it has been found that calreticulin is homologous with the Ral-1 antigen from the filarial nematode *Onchocerca* (Fig. 4). (Unnasch *et al.*, 1988; McCauliffe *et al.*, 1990*b*; Murthy *et al.*, 1990).

FUTURE PROSPECTS

Calreticulin is a major Ca^{2+}-binding (storage) protein in the lumen of the endoplasmic reticulum. However, a number of observations made within the last year are highly suggestive that calreticulin may have more than one cellular function. Of particular interest is recent circumstantial evidence which indicates that calreticulin might be involved in protein–protein interactions within the cell. Guan *et al.* (1991), while examining the properties of the lung flavin-containing mono-oxygenase, discovered that this protein forms a tight complex with calreticulin. Flavin-containing mono-oxygenase catalyses the NADPH-dependent oxidation of drugs, chemicals, and pesticides containing nitrogen, sulphur, and phosphorus (Ziegler, 1988). This enzyme has been identified in both liver and lung tissue in association with endoplasmic reticulum membranes (Ziegler, 1988), and therefore, interactions between calreticulin and flavin-containing mono-oxygenase could be of physiological significance. It is possible, however, that their association is a result of their different charges and occurs only *in vitro* during the protein purification procedure (Guan *et al.*, 1991).

Rojiani *et al.* (1991) used the synthetic peptide KLGFFKR, which represents a highly conserved motif in the cytoplasmic domain of the α-subunit of the integrins, to construct an affinity column for the identification of integrin-binding proteins. Affinity chromatography of cellular extracts, followed by EDTA-dependent elution from the peptide column, resulted in the isolation of a 60-kDa protein identified as calreticulin (Rojiani *et al.*, 1991). Rojiani *et al.* (1991) suggested several potential roles for the interaction between the integrin α subunit and calreticulin. Nevertheless, given that under normal physiological conditions calreticulin is not found in the cytosol, it is difficult to envisage any physiologically relevant interactions between these proteins other than during biosynthesis, transport to the cell surface and/or degradation of the α subunit of the integrin.

An almost identical peptide sequence to the one used by Rojiani *et al.* (1991) is present in a family of steroid receptors (Fig. 6). The amino acid sequence, KxFFKR, which is shorter by one residue than that used by Rojiani *et al.* (1991) (KLxFFKR), is found between the two 'Zn^{2+} finger' motifs of the steroid receptor which are involved in the binding of the receptors to target DNA (Beato, 1989; Luisi *et al.*, 1991). Fig. 6 shows the amino acid sequences of the DNA binding domains of the human steroid hormone receptors (for review see Beato, 1989). The amino acid sequence of the peptide which bound calreticulin (Rojiani *et al.*, 1991) is aligned with the amino acid sequence present in the receptors (Fig. 6). Using mobility-shift assays, it has recently been shown that calreticulin recognizes this peptide sequence in an intact, native steroid hormone receptor and prevents its binding to DNA (K. Burns, B. Duggan, B. Petryk, K. Famulski, R. C. Bleackley & M. Michalak, unpublished work). If calreticulin also interacts with the steroid hormone receptors *in vivo* its role might be one of transporting these molecules to the nucleus (NURP-like function) (Schmitz *et al.*, 1991) or, perhaps, of promoting the dissociation of the receptors from their target DNA. The observation that some calreticulin is localized in the nucleus is relevant to these hypotheses (Opas *et al.*, 1991).

The possibility that calreticulin may have a role in protein–protein interactions within the cell is intriguing. As yet the evidence is preliminary, and the physiological significance of these interactions is unclear. However, it is likely that further studies in this area will contribute significantly to our understanding of the function of calreticulin.

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