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Calreticulin is a candidate for a calsequestrin-like function in Ca^{2+}-storage compartments (calciosomes) of liver and brain

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In a search for the non-muscle equivalent of calsequestrin (the low-affinity high-capacity Ca^{2+}-binding protein responsible for Ca^{2+} storage within the terminal cisternae of the sarcoplasmic reticulum), acidic proteins were extracted from rat liver and brain microsomal preparations and purified by column chromatography. No calsequestrin was observed in these extracts, but the *N*-terminal amino acid sequence of the major Ca^{2+}-binding protein of the liver microsomal fraction was determined and found to correspond to that of calreticulin. This protein was found to bind approx. 50 mol of Ca^{2+}/mol of protein, with low affinity (average K_d approx. 1.0 mM). A monoclonal antibody, C6, raised against skeletal-muscle calsequestrin cross-reacted with calreticulin in SDS/PAGE immunoblots, but polyclonal antibodies reacted with native calreticulin only weakly, or not at all, after SDS denaturation. Immuno-gold decoration of liver ultrathin cryosections with affinity-purified antibodies against liver calreticulin revealed luminal labelling of vacuolar profiles indistinguishable from calciosomes, the subcellular structures previously identified by the use of anti-calsequestrin antibodies. We conclude that calreticulin is the Ca^{2+}-binding protein segregated within the calciosome lumen, previously described as being calsequestrin-like. Because of its properties and intraluminal location, calreticulin might play a critical role in Ca^{2+} storage and release in non-muscle cells, similar to that played by calsequestrin in the muscle sarcoplasmic reticulum.

INTRODUCTION

Eukaryotic cells share the ability to accumulate, within an intracellular membrane-bound compartment, a pool of Ca^{2+} destined to be released rapidly to the cytosol upon activation. In striated-muscle fibres, Ca^{2+} is stored within the sarcoplasmic reticulum in association with calsequestrin, a protein characterized by its high-capacity and low-affinity Ca^{2+}-binding characteristics (MacLennan & Wong, 1971). Calsequestrin is localized within the terminal cisternae and is thought to constitute the major intraluminal Ca^{2+} buffer in the sarcoplasmic reticulum (for review see Endo, 1977; Campbell, 1986). The characteristics of organelles responsible for Ca^{2+} storage in non-muscle cells are still incompletely defined. Subcellular-fractionation experiments have shown that the Ca^{2+}-release activity triggered by Ins(1,4,5)P_3 is recovered in the microsomal fraction (Berridge & Irvine, 1989). The main component of that fraction, the endoplasmic reticulum, was thus proposed to subserve Ca^{2+}-storage and -release functions, similar to those of the sarcoplasmic reticulum in muscle fibres. Subsequent studies have cast doubts on the involvement of the entire endoplasmic reticulum in Ca^{2+} control, and a cytologically distinct structure, the calciosome, has been proposed as the site of regulation of Ca^{2+} in non-muscle cells (Payne & Fein, 1987; Guillemette *et al.*, 1987; Krause & Lew, 1987; Volpe *et al.*, 1988; Oberdorf *et al.*, 1988; Hashimoto *et al.*, 1988; Krause *et al.*, 1989; Rossier *et al.*, 1989; Thevenod *et al.*, 1989).

The expression of a protein that shares properties with calsequestrin has been documented in many non-muscle cell types (Volpe *et al.*, 1988; Hashimoto *et al.*, 1988; Krause *et al.*, 1989; Rossier *et al.*, 1989). High-resolution immunocytochemistry carried out with anti-(skeletal-muscle calsequestrin) antibodies has shown that the calsequestrin-like material resides within the calciosomes (Hashimoto *et al.*, 1988; Volpe *et al.*, 1988). Recent data, however, indicate that calsequestrin is not expressed in non-muscle cells, and interest has focused on other proteins that could play a role in intraluminal Ca^{2+} buffering. One such protein, calreticulin, has recently been cloned and found to include two short sequences similar to sequences in calsequestrin (Fliegel *et al.*, 1989; Smith & Koch, 1989). In the present paper we show that calreticulin is the major Ca^{2+}-binding protein in the rat liver microsomal fraction. On the basis of immunological cross-reactivity and subcellular localization, we conclude that calreticulin corresponds to the calsequestrin-like protein originally described in the calciosome (Volpe *et al.*, 1988).

MATERIALS AND METHODS

Materials

Tris, phenylmethanesulphonyl fluoride, benzamidine, iodoacetamide, leupeptin, pepstatin, aprotinin, Nitro Blue Tetrazolium, 5-bromo-4-chloroindol-3-yl phosphate and alkaline-phosphatase-conjugated rabbit anti-(chicken IgG) antibodies were from Sigma Chemical Co.; EDTA was from Fluka; hydroxyapatite and prestained molecular-mass standards were from Bio-Rad Laboratories; DEAE-cellulose was from Merck; Immobilon membranes were from Millipore; n-octyl glucoside and DNA-labelling kit were from Boehringer Mannheim; $^{45}CaCl_2$ and Hybond were from Amersham International. All other reagents were of the highest available grade.

Subcellular fractionation

Rats and rabbits were killed by decapitation. Livers and brains were rapidly removed and chilled in homogenization buffer

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maintained at 4 °C, trimmed of adherent connective tissues and chopped with scissors into a solution (1:5, w/v) of 240 mM-sucrose supplemented with proteinase inhibitors (1 mM-phenylmethanesulphonyl fluoride and 1 mM-benzamidine). The suspensions were transferred to a Teflon/glass homogenizer, operated either manually or mechanically. Homogenates were centrifuged at 500 *g* for 10 min in a Sorvall SS34 rotor to remove cell debris, nuclei and large membrane sheets. A mitochondria-enriched pellet was obtained by centrifuging the post-nuclear supernatant at 12000 *g* for 20 min, and the post-mitochondrial supernatant was centrifuged at 100000 *g* for 30 min in a Beckman Ti60 rotor to obtain the total microsomal fraction.

Purification of calreticulin

Homogenates or microsomal fractions were suspended in a solution containing 200 g of $(NH_4)_2SO_4$/l at pH 4.7 in the presence of a proteinase inhibitor cocktail (1 mM-phenylmethanesulphonyl fluoride, 1 mM-benzamidine, 1 mM-iodoacetamide, 1 μM-leupeptin, 1 μM-pepstatin and 0.03 trypsin-inhibitory unit of aprotinin/ml) as described by Slupsky *et al.* (1987). Phenylmethanesulphonyl fluoride and benzamidine were included in the solutions used in all subsequent steps of protein isolation. The suspension was centrifuged at 13500 *g* for 30 min in an SS34 rotor, resuspended in 50 ml of 1 mM-EDTA/100 mM-potassium phosphate buffer, pH 7.1, and dialysed against the same buffer overnight at 4 °C. Insoluble material was removed by centrifugation at 13500 *g* for 10 min, and 150–200 mg of supernatant proteins was loaded on to a DEAE-cellulose column (20 cm × 1.5 cm diam.) equilibrated with 1 mM-EDTA/100 mM-potassium phosphate buffer, pH 7.1, containing 50 mM-NaCl. Proteins were eluted with a linear gradient of 0.1–1.0 M-NaCl in the same buffer. A protein of 59 kDa that stained blue with Stains All was eluted at approx. 0.25–0.35 M-NaCl. Fractions containing this protein were identified by SDS/PAGE, pooled and loaded on to a hydroxyapatite column (20 cm × 1.5 cm diam.) equilibrated with 1 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-KCl. Proteins were eluted with a linear gradient (1–200 mM) of phosphate buffer containing 0.2 M-KCl, and the 59 kDa protein was eluted at a phosphate concentration of about 150 mM.

Biochemical analyses

One-dimensional and two-dimensional SDS/PAGE analyses were run according to the procedures of Laemmli (1970) and Michalak *et al.* (1980) respectively. One-dimensional gels were subjected to SDS/7.5%-PAGE at pH 8.8. For two-dimensional gels the first dimension was run at neutral pH (7.0) in 10% disc gels according to the Weber & Osborn (1969) procedure. The disc gels were subsequently equilibrated in Laemmli loading buffer [10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol/2.3% (w/v) SDS/62.5 mM-Tris/HCl buffer, pH 6.8] plus 1% agarose and the second dimension was run on SDS/10%-PAGE gels according to the Laemmli (1970) procedure. In most gels a control lane with sarcoplasmic-reticulum terminal cisternae (R4, according to Saito *et al.*, 1984) was also present, and the apparent molecular masses of the Ca^{2+}-dependent ATPase and of calsequestrin (100 kDa and 64 kDa respectively) were used in addition to the prestained molecular-mass standards from Bio-Rad Laboratories.

Gels were stained by Coomassie Blue, Stains All or the periodic acid/Schiff procedure (Kapitany & Zebrowski, 1973). Blotting of proteins on to nitrocellulose membranes, immunodecoration (Western blotting) and $^{45}Ca^{2+}$ overlay of the blotted proteins were carried out as described previously (Volpe *et al.*, 1988). For microsequencing, proteins were transferred to Immobilon membranes and processed by the conventional techniques (Moos *et al.*, 1988). Equilibrium $^{45}Ca^{2+}$ binding was measured by the use of a continuous-dialysis chamber (Damiani *et al.*, 1989). The medium was 5 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-KCl and 0.1% n-octyl glucoside. Inclusion of the detergent to prevent aggregation and sticking of the protein to the chamber walls was found to be essential. The experiments were performed at 4 °C, either at constant specific radioactivity or by displacing $^{45}Ca^{2+}$ with $^{40}Ca^{2+}$. No significant difference between the two methods was observed. BSA was included in separate wells as the negative control.

Southern and Northern blotting

A rabbit brain cDNA library and rabbit brain polyadenylated RNA preparations were blotted on Hybond membranes and probed with digoxigenin-labelled probes obtained from different segments of rabbit skeletal-muscle calsequestrin cDNA (Maniatis *et al.*, 1982). Southern and Northern blots were washed at low stringency (15 mM-NaCl/1.5 mM-sodium citrate buffer, pH 7, at 52 °C) to detect weakly hybridizing bands, and then developed as described in the labelling kit. A cDNA library of skeletal muscle and skeletal-muscle polyadenylated RNAs were used as positive controls.

Antibodies

Rabbits and hens, used to raise anti-(dog heart calsequestrin) and anti-(rabbit liver 59 kDa protein) antibodies respectively, were injected weekly with 0.1 mg of antigen emulsified with Freund's complete adjuvant and then four more times, at weekly intervals, with Freund's incomplete adjuvant. Total rabbit IgGs were purified from serum (Garvey *et al.*, 1977), and hen IgGs were purified from eggs (Jensenius *et al.*, 1981). Affinity-purified anti-(59 kDa protein) antibodies were prepared as described by Volpe *et al.* (1988). Additional anti-(fast-twitch-skeletal-muscle calsequestrin) antibodies raised in hens were kindly donated by Dr. F. Zorzato and Dr. E. Damiani (Department of Pathology, University of Padova). Monoclonal anti-(skeletal-muscle calsequestrin) antibodies were kindly donated by Dr. E. Zubrzycka-Gaarn (C. H. Best Institute, University of Toronto).

Immunocytochemistry

Small rat liver fragments were excised and immersed directly in a fixative solution containing 2.5% or 4% formaldehyde (freshly prepared from paraformaldehyde) and 0.1% or 0.25% reagent-grade glutaraldehyde in 125 mM-phosphate buffer, pH 7.4. After extensive washing with the buffer, the samples were infiltrated with sucrose and then frozen in Freon 12 cooled with liquid N_2. Ultrathin (50–100 μm thick) cryosections, cut in a Reichart Ultracut microtome equipped with the FC4 apparatus, were recovered over Formvar-coated nickel electron-microscope grids and exposed first to hen anti-(59 kDa protein) antibodies or control IgGs, then to rabbit anti-(chicken IgG) antibodies coating 5 nm-sized gold particles (Janssen, Beerse, Belgium) diluted 1/50. In between these treatments, and after the gold labelling, the grids were extensively washed (6 × 10 min) as described elsewhere (Hashimoto *et al.*, 1988). Immuno-gold-decorated sections were post-fixed with 2% glutaraldehyde in phosphate buffer followed by 0.1% OsO_4 and finally embedded in LR white resin as recommended by Keller *et al.* (1984).

RESULTS

Subcellular fractions

Our first objective was to determine whether low-affinity high-capacity Ca^{2+}-binding proteins could be detected in subcellular fractions from liver and brain. Since most Ca^{2+}-binding proteins bind the carbocyanine dye Stains All to yield metachromatically

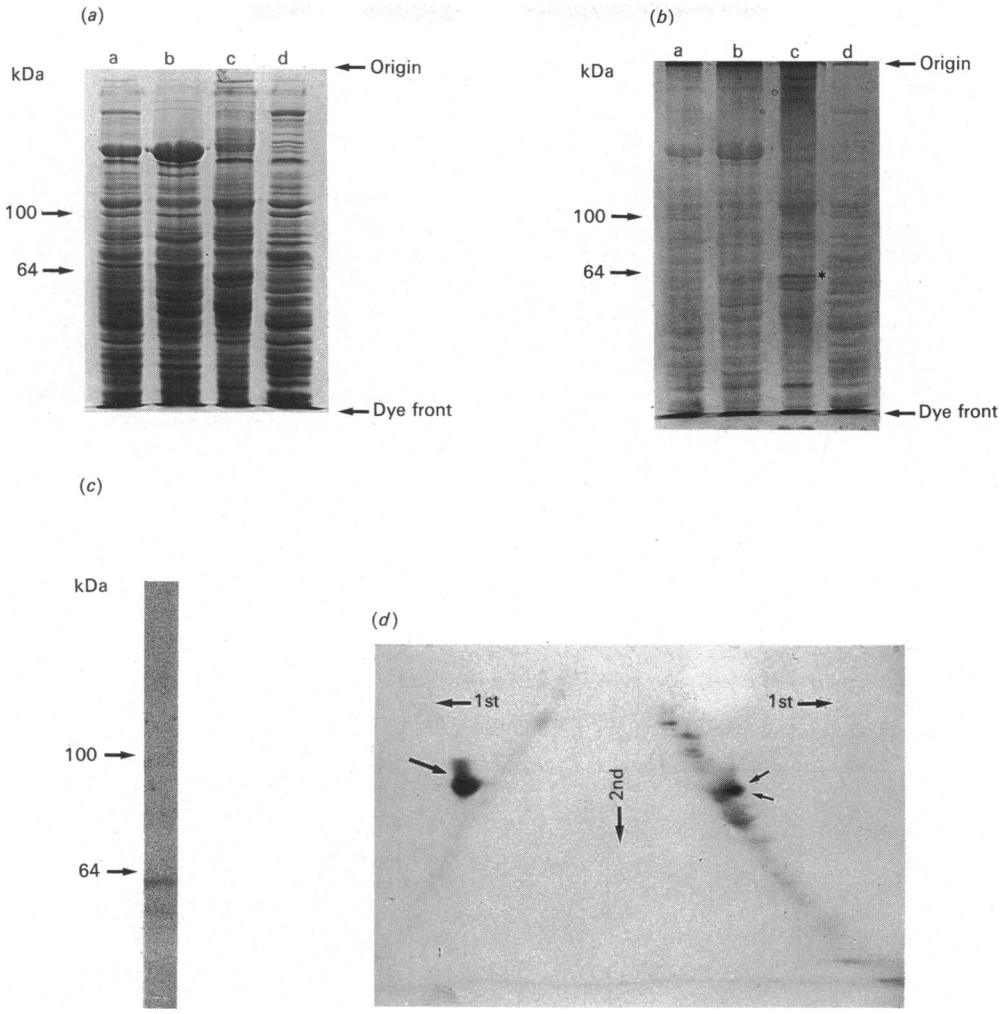

Fig. 1. SDS/7.5%-PAGE of rat liver fractions: lanes a, total homogenate; lanes b, mitochondria-enriched pellet: lanes c, total microsomal fraction; lanes d, 100000 *g* supernatant (100 μg used per lane)

(*a*) Coomassie Brilliant Blue staining. (*b*) Stains All staining: the strong labelling at the top of the gel is probably due to RNA, since it disappears after incubation with RNAase. The 59 kDa metachromatic band appears in lane c as a darker band and is indicated by an asterisk (*). (*c*) $^{45}Ca^{2+}$ overlay of lane c. (*d*) Coomassie Brilliant Blue staining of a two-dimensional SDS/10%-PAGE [according to the Michalak *et al.* (1980) procedure] of the liver microsomal fraction and of the sarcoplasmic-reticulum fraction R4. The large arrow indicates skeletal-muscle calsequestrin. The two small arrows indicate calreticulin.

blue-stained complexes (Cala & Jones, 1983), SDS/PAGE-separated proteins from post-nuclear supernatant, mitochondria-enriched, total microsomal and 100000 *g* supernatant fractions were stained with the dye, and the resulting patterns (Fig. 1*b*) were compared with those obtained after Coomassie Blue staining of identical fractions (Fig. 1*a*). Although metachromatically blue-stained proteins were present in all liver fractions, a major blue-stained band with an apparent molecular mass of 59 kDa was present almost exclusively in the microsomal fraction (Fig. 1*b*). The microsomal fraction was also studied by two-dimensional SDS/PAGE according to the Michalak *et al.* (1980) procedure (pH 7 in the first dimension, pH 8.8 in the second dimension). The 59 kDa band moved off the diagonal, indicating a slower migration at alkaline than at neutral pH (Fig. 1*d*, right). This pH-dependency of electrophoretic mobility is a typical characteristic of skeletal-muscle calsequestrin (Fig. 1*d*, left) and has been attributed to the fact that at alkaline pH the negative charge of calsequestrin is increased, resulting in a more asymmetric structure (Cozens & Reithmeier, 1984).

When the microsomal proteins separated by SDS/PAGE were blotted on to nitrocellulose and subjected to $^{45}Ca^{2+}$ overlay, the 59 kDa protein was the most prominent Ca^{2+}-binding band (Fig. 1*c*).

The 59 kDa protein content was quantified by microdensitometry of the blue band, based on a standard curve constructed with increasing amounts of the purified protein. The results indicate that the band accounts for 1–2% of the total liver microsomal protein. In order to establish the localization of the 59 kDa protein in the microsomal membranes, the fraction was treated as follows: (i) washed with 0.6 M-KCl for 1 h, to release peripheral proteins; (ii) subjected to alkaline extraction at low osmotic strength, to release the luminal content; (iii) treated with 1% Triton X-100 or Nonidet P40, to dissolve the membranes. Washing with the high-salt solution did not result in appreciable solubilization of the 59 kDa protein, whereas most of it was extracted by the other two procedures (results not shown), demonstrating that the protein is localized in the luminal compartment.

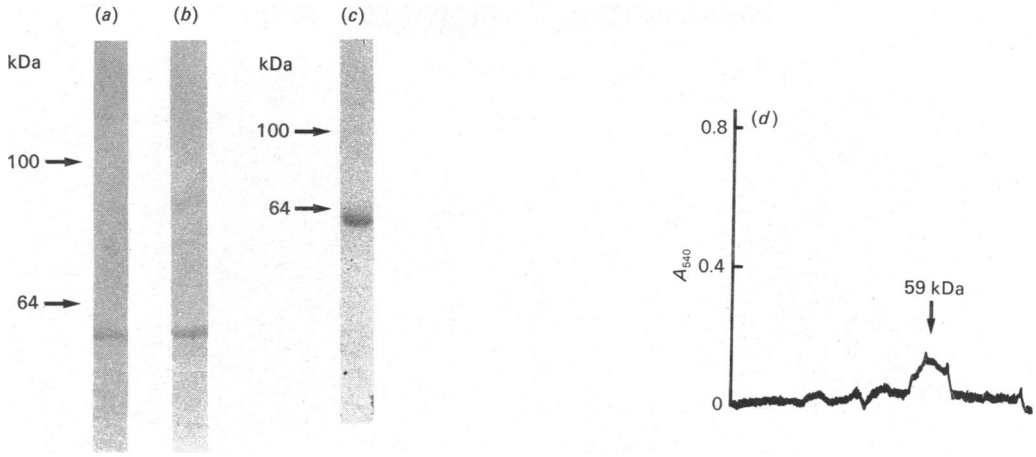

Fig. 2. SDS/7.5%-PAGE of purified calreticulin

(*a*) Coomassie Brilliant Blue staining, (*b*) Stains All staining and (*c*) $^{45}Ca^{2+}$ overlay (30 μg of protein in each lane); (*d*) densitometric scanning at 540 nm after periodic acid/Schiff staining (180 μg of protein).

Table 1. *N*-Terminal amino acid sequences of calreticulins purified from rat liver, rabbit liver (59 kDa and 61 kDa) and rabbit brain

The bottom line shows the published amino acid sequence for rabbit liver calregulin (Khanna *et al.*, 1987*a*).

Ca^{2+}-binding protein	*N*-Terminal sequence
Rat liver calregulin	Asp-Pro-Ala-Ile-Tyr-Phe-Lys-Glu-Gln-Phe-Leu-Asp-
Rabbit liver calreticulin (59 kDa)	Glu-Pro-Val-Val-Tyr-Phe-Lys-Glu-Gln-Phe-Xaa-Asp-Gly-Asp-Gly-Xaa-Xaa-Glu-Arg-
Rabbit liver calreticulin (61 kDa)	Glu-Pro-Val-Val-Tyr-Phe-Leu-Glu-Gln-Phe-Leu-Asp-Gly-Asp-Gly-Xaa-Thr-Glu-Arg-
Rabbit brain calreticulin	Pro-Val-Val-Tyr-Phe-Lys-Glu-Gln-Phe-Leu-Asp-Gly-Asp-Gly-
Rabbit liver calregulin	Glu-Pro-Val-Val-Tyr-Phe-Lys-Glu-Gln-Phe-Leu-Asp-Gly-Asp-Gly-

Purification and characterization of the 59 kDa protein

In previous studies, calsequestrin and the high-affinity Ca^{2+}-binding protein were purified from muscle sarcoplasmic reticulum by column chromatography (MacLennan & Wong, 1971; Ostwald & MacLennan, 1974; Slupsky *et al.*, 1987). Similar techniques were now employed to purify the 59 kDa protein from the rat liver microsomal fraction. In Figs. 2(*a*) and 2(*b*) we compare the Coomassie Blue and Stains All staining of the purified protein. Only minor contaminants were found to be present. $^{45}Ca^{2+}$ overlay experiments (Fig. 2*c*) demonstrated that the 59 kDa protein accounts for practically all the Ca^{2+} binding in the preparation. In addition, the 59 kDa protein was found to stain with the periodic acid/Schiff reagents (Fig. 2*d*), revealing its glycoprotein nature.

For molecular identification, *N*-terminal microsequencing was carried out on the 59 kDa protein isolated from rabbit liver and brain and rat liver, blotted on Immobilon membranes (Table 1), and on a 61 kDa protein that also stained blue with Stains All. The *N*-terminal sequences of the 59 kDa proteins from the various sources were similar to or identical with that of calregulin (Khanna *et al.*, 1987*a*), the high-affinity Ca^{2+}-binding protein of the muscle sarcoplasmic reticulum (Fliegel *et al.*, 1989), and CRP55, from mouse liver (Smith & Koch, 1989). The 61 kDa protein from rabbit liver is of considerable interest. Although it had an *N*-terminal sequence similar to that of the 59 kDa protein, the mobility of the protein in SDS/PAGE was clearly different and the protein contained a leucine-for-lysine substitution at position 7. Accordingly, it could be an isoform of the 59 kDa protein derived from a different gene.

Ca^{2+} binding to the 59 kDa protein

The Ca^{2+}-binding ability of the 59 kDa protein was quantified, at equilibrium, in a continuous-dialysis apparatus. In our hands the 59 kDa protein was found to be highly sensitive to proteolytic degradation. The integrity of the analysed protein was therefore systematically checked by SDS/PAGE at the end of the long equilibrium-binding experiments. In Fig. 3(*a*) we illustrate the Ca^{2+}-concentration-dependence for Ca^{2+} binding in the presence of 0.1 M-KCl at pH 7.5. Between 2.5 μM and 2 mM, the Scatchard plot was clearly not monophasic (Fig. 3*b*). $B_{max.}$ was found to approach 1 μmol of Ca^{2+}/mg of protein, corresponding to approx. 50 mol of Ca^{2+}/mol of protein. Half-maximal saturation was observed at around 1.0 mM-Ca^{2+}. Deviation from linearity at low Ca^{2+} concentrations revealed the occurrence of a high-affinity Ca^{2+}-binding site, but binding below 100 μM-Ca^{2+} was not investigated in detail. In order to evaluate the contribution of the 59 kDa protein to the total luminal Ca^{2+}-binding capacity of the microsomal fraction, 1%-Nonidet P40 extracts of the fraction were analysed by equilibrium dialysis under conditions identical with those employed for the purified protein. At 1 mM free Ca^{2+}, a total binding of 45 nmol/mg of protein was observed; since the 59 kDa protein accounted for 5% of the proteins in the extract, 57% of this binding could be attributed to the 59 kDa protein on the basis of a K_d of 1 mM and a $B_{max.}$ of 50 mol/mol, assuming

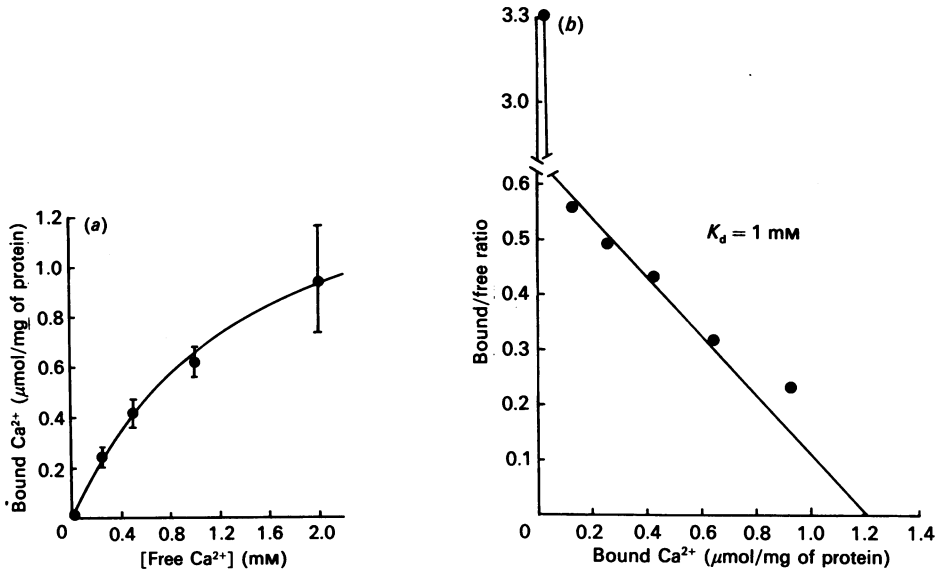

Fig. 3. (*a*) Ca^{2+} binding to purified calreticulin and (*b*) Scatchard plot of $^{45}Ca^{2+}$ binding to calreticulin

Conditions are described in the Materials and methods section. The results shown are means ± S.D. for three independent experiments.

no interference by the other proteins. This result is in agreement with the data obtained from the $^{45}Ca^{2+}$ overlay, which indicated that the 59 kDa protein is the major Ca^{2+}-binding protein in the liver microsomal fraction.

Antigenic characteristics of the 59 kDa protein

Northern and Southern blots did not reveal expression of muscle calsequestrin in liver or brain (results not shown); these findings are complementary to the results obtained by Scott *et al.* (1988), which showed no expression of the cardiac isoform of calsequestrin in brain. In order to explain results obtained previously with anti-calsequestrin antibodies, we first looked for cross-reactivity between calsequestrin and the 59 kDa protein by using Western blotting. We tested nine anti-calsequestrin antibodies [four monoclonal and four polyclonal anti-(skeletal-muscle calsequestrin) antibodies and one polyclonal anti-(cardiac calsequestrin) antibody] and found all of them but one to react weakly, or not at all, with the 59 kDa protein (results not shown). The monoclonal anti-calsequestrin antibody C6 reacted well with both calsequestrin and the 59 kDa protein. Affinity-purified antibodies raised against the 59 kDa protein reacted strongly with their purified antigen and with a single band of the same molecular mass in the microsomal fraction, but failed to recognize muscle calsequestrin.

Immuno-dot-blot experiments in which the proteins were exposed to the different antibodies in their native state or after different pretreatments were also carried out. As shown in Fig. 4, the native 59 kDa protein reacted strongly with the same anti-(fast-twitch-skeletal-muscle calsequestrin) antibody (dot a), previously used to identify the calciosome (Volpe *et al.*, 1988). Additional non-related proteins such as carbonic anhydrase and RNAase (Fig. 4, dots b and c) remained negative. It may be noted that the cross-reactivity of the 59 kDa protein was maintained when the dotted protein was pretreated with an aldehyde fixative mixture (4% formaldehyde and 0.1% glutaraldehyde in 125 mM-phosphate buffer, pH 7.4) that is used in the processing of cells and tissues for cryosection immunolabelling (results not shown). In contrast, when dotted proteins were pretreated with SDS (0.1% in 125 mM-phosphate buffer, pH 7.4) the cross-immunoreactivity of the 59 kDa protein with anti-calsequestrin

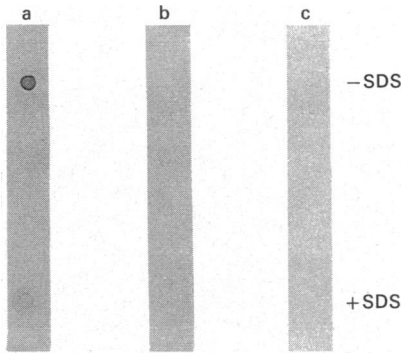

Fig. 4. Immunoenzymic staining of purified native and SDS-denatured calreticulin

Samples (5 μg) of protein, native or treated with 1% SDS, were placed on nitrocellulose membranes; immunoenzymic staining was with specific anti-(skeletal-muscle calsequestrin) antibodies. Dots a, calreticulin; dots b, carbonic anhydrase; dots c, RNAase.

antibodies was largely lost, thus explaining the negative Western-blotting results reported by Van *et al.* (1989).

Immunocytochemistry

Liver ultrathin cryosections were immunodecorated with small (5 nm in diameter) colloidal gold particles. With appropriate concentrations of affinity-purified anti-(59 kDa protein) antibodies (Fig. 5), the labelling of the cytosol and most subcellular structures (nuclei, mitochondria, Golgi cisternae, multivesicular bodies, plasma membrane) was very low, close to the background levels observed when either control IgG was used or the first antibody was omitted (< 2 particles/μm²). Under these conditions small gold-particle clusters were observed over the lumen of small (approx. 100 nm in diameter) profiles, distributed throughout the cell, often in the proximity of, or in close apposition to, endoplasmic-reticulum cisternae (arrows in Fig. 5). The limiting membrane of these structures was appropriately resolved when cryosections were either cut perpendicularly or of considerable thickness (see Fig. 5*c*). All these properties of the

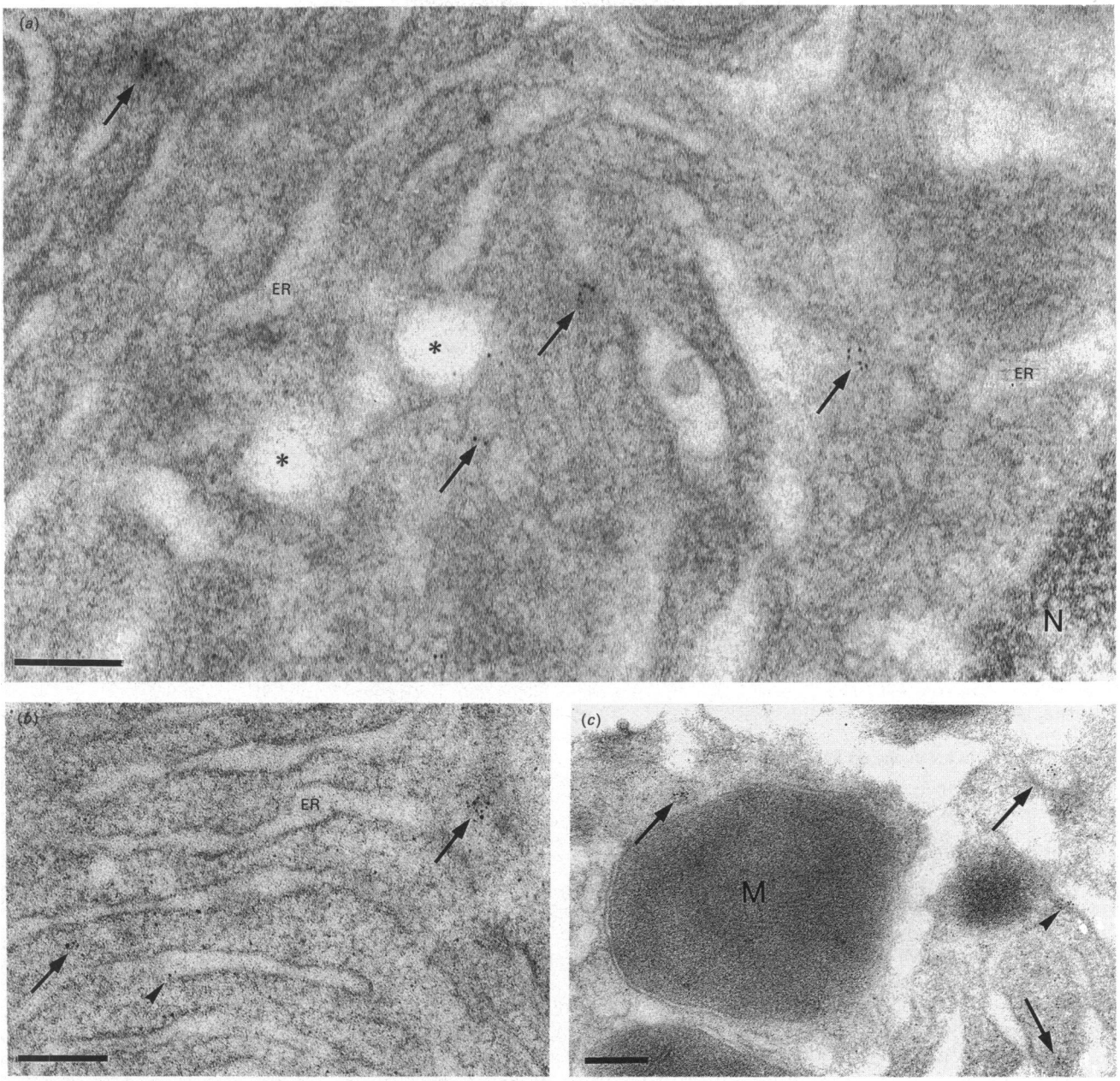

Fig. 5. Anti-calreticulin immuno-gold labelling of liver ultrathin cryosections

Immunoreactivity is concentrated primarily over small vacuolar profiles (approx. 100 nm in diameter, arrows). The limiting membrane of these structures is better resolved in the thicker cryosection shown in (*c*). Mitochondria (M), the nucleus (N) and the nuclear envelope appear negative. Many endoplasmic-reticulum (ER) cisternae are also unlabelled; two, however, exhibit a small amount of gold particles (arrowheads in *b* and *c*). Asterisks (*) indicate small artifactual swellings of the frozen tissue. The horizontal bars represent 0.2 μm.

59 kDa-protein-positive structures coincide with those previously reported for calciosomes, originally identified by their immuno-gold labelling with anti-calsequestrin antibodies (Volpe *et al.*, 1988; Hashimoto *et al.*, 1988). In the present work, additional gold particles were scattered within the lumen of recognizable rough-surfaced endoplasmic-reticulum cisternae (arrowheads in Fig. 5). In order to quantify the distribution of the antigen among calciosomes and endoplasmic reticulum, accurate counts (see Hashimoto *et al.*, 1988) were made in a group of 18 randomly selected pictures exhibiting good preservation of the liver ultrastructure, which were printed at the same final magnification ($\times$ 50000). Out of 755 gold particles counted, 617 were within 96 small profiles and 138 within 385 endoplasmic-reticulum-cisternal profiles. In these pictures the percentage of the total surface area occupied by the former structures was 1.1%, a value similar to that reported for calciosomes (Hashimoto *et al.*, 1988); the area of the endoplasmic-reticulum cisternae was 18%. Specific immunolabelling was therefore approx. 600 and 6 gold particles/μm^2 respectively.

DISCUSSION

On the basis of the results reported in the present paper (molecular mass, metachromatic staining, Ca^{2+} binding and *N*-terminal sequence), the 59 kDa protein of liver and brain microsomal fractions can be identified as calreticulin, an acidic Ca^{2+}-binding protein present in most mammalian tissues. Thus the 59 kDa protein is now referred to below as calreticulin. The

abundance of calreticulin in a microsomal fraction endowed with Ins(1,4,5)P_3-sensitive Ca^{2+} release and its ability to bind Ca^{2+} with high capacity and low affinity make this protein a likely candidate for intravesicular Ca^{2+} buffering. In this respect calreticulin could be the functional non-muscle equivalent of calsequestrin, the low-affinity high-capacity Ca^{2+}-storage protein of the striated-muscle sarcoplasmic reticulum (MacLennan & Wong, 1971; Endo, 1977; Campbell, 1986). Previous studies on calreticulin demonstrated the existence of one high-affinity Ca^{2+}-binding site (1 mol of Ca^{2+} bound/mol of protein; K_d in the low micromolar range) (Ostwald & MacLennan, 1974; Waisman *et al.*, 1985; Khanna *et al.*, 1987*b*; Tokuda *et al.*, 1987). In addition, muscle calreticulin was reported to bind numerous Ca^{2+} ions with low affinity (approx. 25/mol; Ostwald & MacLennan, 1974). In our present equilibrium $^{45}Ca^{2+}$-binding experiments, the number of low-affinity sites in liver calreticulin was found to be approx. 50/mol of protein. The difference with respect to the data obtained by Ostwald & MacLennan (1974) for the muscle protein probably depends on the inclusion of detergent in our experimental conditions. This result concurs with others (Damiani *et al.*, 1989; Macer & Koch, 1989), but contrasts with that reported by Van *et al.* (1989), who failed to reveal any low-affinity sites in rat liver calreticulin. It should be noted, however, that in the latter study the number of sites for muscle calsequestrin, investigated in parallel, was also much lower than that reported in the literature by all other groups.

Our fractionation and immunocytochemistry experiments demonstrate that calreticulin is localized within the lumen of membranous structures. Consistent with this conclusion, muscle calreticulin has been demonstrated to be synthesized with a signal sequence and to contain no transmembrane domain (Fliegel *et al.*, 1989). Because of its high tissue concentration, Ca^{2+}-binding characteristics and subcellular localization, calreticulin can be expected to play a key role in the control of Ca^{2+} homoeostasis in non-muscle cells. We estimate that in liver and brain the protein accounts for 1–2% of the total microsomal protein and, in the liver microsomal fraction, for approx. 60% of the luminal Ca^{2+} buffering. On the basis of morphometric data (Weibel *et al.*, 1969), the maximum Ca^{2+} binding by the protein can thus be calculated to be approx. 0.5–1 mmol/l of cytosol, an amount that appears sufficient to sustain the $[Ca^{2+}]_i$ transients (in the micromolar range) originated from intracellular stores.

Immunological and high-resolution immunocytochemical results indicate that liver calreticulin is concentrated within vacuolar profiles indistinguishable, in size, shape and distribution, from calciosomes, where it corresponds to the calsequestrin-like protein initially employed as identification marker. In fact, the polyclonal anti-calsequestrin antibody, originally used to identify calciosomes, reacted in dot-blot assays with the native and aldehyde-fixed calreticulin; the same occurred with one out of the four monoclonal anti-calsequestrin antibodies that we tested. The two proteins appear therefore to express common epitopes, a conclusion that is also consistent with the report of stretches of similar amino acid sequences (Smith & Koch, 1989).

As yet, calciosomes have not been isolated as a pure subcellular fraction, and their biochemistry is still largely unknown. As discussed in detail elsewhere (Hashimoto *et al.*, 1988; Volpe *et al.*, 1988), the structure of calciosomes could be envisaged as either a discrete organelle or a specialized expansion, luminally continuous with endoplasmic-reticulum cisternae. This latter possibility might be consistent with the report that in cerebellar Purkinje cells the Ins(1,4,5)P_3 receptor (an expected component of the calciosome; see Volpe *et al.*, 1988) is concentrated in a set of clearly specialized membranes that are continuous with, and represent therefore a specialized subcompartment of, the endoplasmic reticulum (Satoh *et al.*, 1990). In addition, labelling with anti-calreticulin antibodies (low, but above the background) was observed, in the present work, within some rough-surfaced cisternae. In this respect, it may be added that calreticulin cDNA sequencing data predict the protein to include the tetrapeptide Lys-Asp-Glu-Leu at the *C*-terminus (Fliegel *et al.*, 1989; Smith & Koch, 1989). This sequence is typical of many cisternal proteins, which are prevented or retarded from being transported to the Golgi complex, and therefore tend to reside within the endoplasmic reticulum (Munro & Pelham, 1987; Zagouras & Rose, 1989).

On the other hand, calreticulin from rat liver is a glycoprotein, as revealed by its positive reaction with the periodic acid/Schiff reagents. The analyses by Van *et al.* (1989) have revealed that the sugar chains of rat liver calreticulin are processed up to the addition of terminal galactose, an event known to occur only in the trans-Golgi network (Roth & Berger, 1982; Farquhar, 1985), a distal section of the secretory pathway from which re-routing to the endoplasmic reticulum has never been reported. If confirmed, the data of Van *et al.* (1989) would therefore be consistent with a passage of calreticulin through the Golgi apparatus to the calciosome compartment. Additional studies, at both the molecular and the cellular level, appear to be needed in order to solve this question.

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