Overexpression of Calsequestrin in L6 Myoblasts: Formation of Endoplasmic Reticulum Subdomains and Their Evolution into Discrete Vacuoles Where Aggregates of the Protein Are Specifically Accumulated

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Calsequestrin (CSQ), the major low-affinity Ca^{2+}-binding glycoprotein of striated muscle fibers, is concentrated to yield aggregates that occupy the lumen of the terminal cisternae of the sarcoplasmic reticulum (SR). When infected or transfected into L6 myoblast, the protein is also concentrated, however, in dense vacuoles apparently separate from the endoplasmic reticulum (ER). CSQ-rich cells appear otherwise normal; in particular, neither other proteins involved in Ca^{2+} homeostasis nor ER chaperones are increased. The CSQ dense vacuoles are shown herein to be specialized ER subdomains as demonstrated by 1) the endoglycosidase H sensitivity of their CSQ and 2) two markers, calreticulin and calnexin (but not others, protein disulfide isomerase and BiP), intermixed with the vacuole content. Their formation is shown to start with the aggregation of CSQ at discrete sites of the ER lumen. When cells were transfected with both CSQ and calreticulin, only the first gave rise to vacuoles; the second remained diffusely distributed within the ER lumen. The possibility that CSQ aggregation is an artifact of overexpression appears unlikely because 1) within dense vacuoles CSQ molecules are not disulfide cross-linked, 2) their turnover is relatively slow ($t = 12$ h), and 3) segregated CSQ is bound to large amounts of Ca^{2+}. Transfection of a tagged CSQ into cells already overexpressing the protein revealed the continuous import of the newly synthesized protein into preassembled vacuoles. The tendency to aggregation appears, therefore, as a property contributing to the segregation of CSQ within the ER lumen and to its accumulation within specialized subdomains. The study of L6 cells expressing CSQ-rich vacuoles might thus ultimately help to unravel mechanisms by which the complexity of the sarcoplasmic reticulum is established in muscle fibers.

INTRODUCTION

In recent years, considerable attention has been devoted to the mechanisms by which soluble proteins, after synthesis by membrane-bound polysomes, are retained within the lumen of the endoplasmic reticulum (ER)[1] instead of embarking along the secretory pathways. The identification of C-terminal tetrapeptides, KDEL

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[1] Abbreviations used: AVP, arginine vasopressin; BiP, Ig heavy chain-binding protein; BFA, brefeldin A; CD, cathepsin D; CNX, calnexin; CRT, calreticulin; CSQ, calsequestrin; DAMP, 3-(2,4-dinitroanilino)-3'amino-N-methyl-dipropylamine; Endo-H, endoglycosidase H; ER, endoplasmic reticulum; GC, Golgi complex; IONO, ionomycin; IP_3, myo-inositol 1,4,5-trisphosphate; lgp120, lysosomal membrane glycoprotein of 120 kDa; PDI, protein disulfide isomerase; PVP, polyvinylpyrrolidone; SERCA, sarcoplasmic endoplasmic reticulum Ca^{2+} ATPases; SR, sarcoplasmic reticulum; TG, thapsigargin.

and homologues, and of their membrane receptor system shuttling from the Golgi complex (GC) to the ER and vice versa has elucidated a dynamic process, i.e., continuous recycling (Munro and Pelham, 1987; Lewis and Pelham, 1992), that accounts, at least in part, for the residency of various such proteins (see Nilsson and Warren, 1994, and references therein). This picture, however, cannot be applied to all ER lumenal proteins because a group of them does not exhibit the C-terminal KDEL sequence. The mechanisms of this group have remained so far largely unknown.

Among the ER lumenal proteins devoid of KDEL is calsequestrin (CSQ), the major low-affinity high-capacity Ca^{2+}-binding protein of striated muscle fibers (Fliegel *et al.*, 1987; Clegg *et al.*, 1988; Scott *et al.*, 1988). The distribution of this protein appears peculiar. In both skeletal and heart fibers, CSQ does not distribute widely along the ER, as occurs with at least some KDEL proteins, but is concentrated into dense aggregates that fill the lumen of a sarcoplasmic reticulum (SR) subcompartment, the terminal (in the heart also corbular) cisternae (Jorgensen *et al.*, 1983; Franzini-Armstrong *et al.*, 1987; Pozzan *et al.*, 1994). Such a distribution has physiological significance because it provides large amounts of the cation adjacent to ryanodine receptors, to be released upon stimulation to sustain muscle contraction. Nonrandom distribution of CSQ within the ER has been observed also in the other cell types where expression of the protein has been reported, although to a degree much lower than in muscle fibers, for example in rat smooth muscle fibers and avian cerebellar Purkinje neurons (Wuytack *et al.*, 1987; Villa *et al.*, 1991, 1993b; Volpe *et al.*, 1991).

To explain their heterogeneous distribution within the muscle SR, CSQ molecules were proposed to be bound (docked) by two families of basic membrane proteins, the triadins and junctins (M_r = ~95 and 30 kDa). These proteins, first identified by overlay binding (Mitchell *et al.*, 1988; Damiani and Margreth, 1990), were then cloned and shown to be located in the ryanodine receptor-rich areas of the SR membranes (Caswell *et al.*, 1991; Knudson *et al.*, 1993; Guo *et al.*, 1994, 1996; Jones *et al.*, 1995). Whether docking alone accounts for the localization of CSQ is, however, still unclear. In this respect, it is worth mentioning that during muscle development CSQ aggregates have been observed within ample areas of the SR, including longitudinal cisternae and also discrete vesicles apparently in transit toward their final destination (Flucher, 1992; Villa *et al.*, 1993a). Moreover, in smooth muscle fibers and avian Purkinje neurons, expression of triadins and junctins has never been reported. We conclude that CSQ localization may well be a complex process, various events of which, such as the blockade of traffic to the GC and the formation of protein aggregates, have not been explained yet.

The approach developed in the present work was based on the infection/transfection of L6 rat myoblasts (Yaffe, 1973) with the cDNA specific for the CSQ from two species, the chicken and, in some experiments also, the rabbit. Previous studies in this laboratory have revealed that, within the growing cells of L6 clones stably overexpressing CSQ, the Ca^{2+}-binding protein is not distributed throughout the ER but largely concentrated within large, dense, and apparently discrete vacuoles (Raichman *et al.*, 1995). The latter were interpreted as post-GC organelles on their way to be degraded by crinophagy based on three criteria, the first of which, exclusion of the ER marker protein disulfide isomerase (PDI), was common to all CSQ-rich vacuoles and the other two criteria, lumenal acidity and labeling for the lysosomal enzyme cathepsin D (CD), appeared in only a fraction of the vacuoles (Raichman *et al.*, 1995). The present study, however, demonstrates that, in contrast to the previous conclusion, the CSQ-rich vacuoles of L6 myoblasts are ER in nature and constitute, therefore, specialized domains distinct from the rest of the endomembrane system. In addition, these vacuoles exhibit similarities with the organelles rich in endogenous CSQ typical of striated muscle fibers and other cells and could, therefore, be envisaged as an experimental model in which properties of the Ca^{2+}-binding protein could be investigated in a cellular environment.

MATERIALS AND METHODS

Materials

The L6 myogenic cell line, developed by Yaffe (1973), was purchased from American Type Culture Collection (Rockville, MD). The clones primarily used in this study, generated by infection (Clegg *et al.*, 1988; Muller *et al.*, 1990) and characterized as described previously (Raichman *et al.*, 1995), were the control (mock cells) C1 and the CSQ overexpressor A2. The latter largely resembles clone A3 used by Raichman *et al.* (1995).

The antibodies (Abs) used have been described elsewhere: anti-CSQ, a rabbit polyclonal Ab, Hall *et al.* (1988); anti-calreticulin (CRT), a rabbit polyclonal Ab, Perrin *et al.* (1991); anti-PDI C-terminal tail (ID3), a mouse monoclonal Ab, Vaux *et al.* (1990); anti-lysosomal membrane protein (lgp120), a rabbit polyclonal Ab, Lewis *et al.* (1985); anti-CD, a rabbit polyclonal Ab, Hashimoto *et al.* (1988); anti-mannosidase II, a rabbit polyclonal Ab, Velasco *et al.* (1993); anti-calnexin (CNX), a rabbit polyclonal Ab, Wada *et al.* (1994); anti-BiP, a rat monoclonal Ab, Bole *et al.* (1986); anti-sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPases (SERCA), a mouse monoclonal Ab, Colyer *et al.* (1989); anti-*myo*-inositol 1,4,5-trisphosphate (IP_3) receptors, a rabbit polyclonal Ab, Peng *et al.* (1991). The monoclonal Ab against the HA1 tag of hemagglutinin (monoclonal Ab 12CA5) was purchased from the Berkley Antibody (Berkley, CA); anti-β-galactosidase, a mouse monoclonal Ab, was from Boehringer-Mannheim Italia (Milan, Italy); anti-3-(2,4-dinitroanilino)-3'-amino-N-methyl-dipropylamine (DAMP), a mouse monoclonal Ab, and its antigen DAMP were from Oxford Biomedical Research (Oxford, MI).

Rhodamine-, fluorescein-, and indodicarbocyanine (Cy5)-labeled sheep IgGs against rabbit and mouse IgGs and unlabeled goat Fab

fragments and goat serum were purchased from Technogenetics (Milan, Italy). Colloidal gold particles (5 and 15 nm), coated with goat IgGs against either rabbit or mouse IgGs, were purchased from Biocell (Cardiff, United Kingdom). Before each labeling experiment, the colloidal gold preparations were carefully checked in the electron microscope for adherence to specifications in terms of both size uniformity and absence of aggregates. Only the preparations exhibiting >95% of single particles and no aggregates larger than two particles were used in the present work. Other chemicals were analytical or the highest grade available. Protein A-Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden); brefeldin A (BFA) was from Sigma-Aldrich (Milan, Italy); the ECL Western blotting detection reagent and PRO-MIX:L-[^{35}S] for in vitro cell labeling was from Amersham (Buckinghamshire, United Kingdom); culture sera and media were from GIBCO (Basel, Switzerland); the remaining chemicals were from Sigma-Aldrich.

Cell Culture and Transient Transfections

C1 and A2 clones of L6 myoblast cells were grown at 37°C in a 5% CO_2 in DMEM supplemented with 15% fetal calf serum and 100 U/ml penicillin and streptomycin. Purified plasmid containing the chicken CSQ cDNA (pLNLCMV-CAL), the same used for stable infection of cells (Muller et al., 1990), was introduced by transfection into attached growing L6 cells by using polyethylenimine (Boussif et al. 1995), a protocol by which the percentage of transfected cells (~10%) was distinctly higher than with calcium phosphate and Lipofectin. Additional experiments were carried out with C1 and A2 clone cells transiently transfected with the cDNA of either the human CRT or the rabbit CSQ, both tagged with the hemagglutinin HA1 sequence and inserted in the expression plasmid pcDNAI as described (for CRT) by Bastianutto et al. (1995). The day before transfection the cells were seeded to 40–50% confluence with minimal cell–cell contacts. The cDNA used for a 10-mm Petri dish was 30–40 μg.

Immunocytochemistry

Cell culture monolayers were fixed for 15 min at 4°C with 4% paraformaldehyde and 0.25% glutaraldehyde in 125 mM phosphate buffer and washed with the same buffer. For immunofluorescence (light and confocal microscope), cells were then treated for 30 min with a solution containing 0.3% Triton X-100, 15% filtered goat serum, 0.45 M NaCl, and 10 mM phosphate buffer, pH 7.4. After washing, the preparations were exposed (90 min at 37°C or overnight at 4°C) to any one of the various primary Abs diluted in the above Triton X-100 and goat serum-containing solution. After an additional thorough wash, the cells were treated with the appropriate rhodamine- or fluorescein-labeled sheep Ab (1:100–1:200 dilution in the Triton X-100–goat serum solution, 60 min, 37°C), washed again, and mounted in glycerol to be examined with a laser scanning confocal microscope MRC 1024, Bio-Rad House (Hemel Hempstead, Hertfordshine, United Kingdom). For double and triple labeling, the above procedure was repeated in sequence, and the secondary Abs were those coupled to rhodamine and fluorescein mentioned above, as well as another Ab coupled to indodicarbocyanine. When primary Abs from different species (mouse and rabbit) were used, no signal spill-over was observed, whereas with two rabbit Abs some spill-over was present. To take care of the problem, these experiments were systematically run in duplicate and the order of the primary Abs was switched in the two parallel samples.

The cells of monolayers destined to be processed for immunoelectron microscopy were detached by trypsin treatment, washed, and centrifuged at low speed. For cryosections, the pellets, after fixation for 1 h with the 4% paraformaldehyde and 0.25% glutaraldehyde mixture and washing as above, were infiltrated in 0.6 M sucrose mixed with 7% polyvinylpyrrolidone (PVP) and then brought to 1.86 M sucrose and 20% PVP by successive increases of

the infiltrating solution concentrations. Freezing was in a 3:1 mixture of propane and cyclopentane cooled with liquid nitrogen. Ultrathin sections (40–80 nm) were cut with a Ultracut ultramicrotome equipped with a FC4 cryosection apparatus (both from Reichert-Jung, Vienna, Austria), collected over nickel grids, and covered with 2% gelatin. After treatment with 125 mM sodium phosphate buffer supplemented with 0.1 M glycine, pH 7.4, they were exposed for 90 min at 37°C to the first Ab diluted in phosphate-glycine buffer, then washed with the buffer, and finally labeled with anti-IgG-coated gold particles (5 or 15 nm, dilution 1:100 in the same buffer). For dual labeling, the sections were exposed in sequence to the two Abs, followed by the corresponding gold particles, with appropriate washing and quenching steps in between (see Villa et al., 1993b). The samples used for water-soluble acrylic resin embedding were fixed with the aldehyde mixture, then detached, and recovered by centrifugation as for cryosections. After extensive washes with phosphate buffer, the pellets were dehydrated in ethanol and embedded in LR White (Newman, 1989). Ultrathin sections were collected over nicked grids, exposed for 90 min at 37°C to the anti-CSQ Ab diluted in phosphate-glycine buffer, and then labeled with anti-IgG-coated gold particles. After washing, the grids were postfixed with 1% glutaraldehyde in phosphate buffer and then stained with uranyl acetate and lead citrate.

^{45}Ca Measurements

C1 and A2 clone cells were grown as described above (Ca^{2+} concentration in the medium was 1.8 mM), except that during the last 48 h, their incubation medium was supplemented with $^{45}Ca^{2+}$ (4 μCi/ml). At the end of this period, the labeled cells were trypsinized, centrifuged (300 × g for 5 min), and then rapidly washed twice in Krebs-Ringer-N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) medium (containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, and 25 mM HEPES-NaOH, pH 7.4). The cells were then quickly resuspended at 37°C in 4.4 mM EGTA-containing Ca^{2+}-free medium, and a mixture of vasopressin (AVP) at 1 μM and thapsigargin (TG) at 0.13 μM, in dimethyl sulfoxide was added. Further additions, similarly made at 4 min distance, were of ionomycin (IONO, 1 μM in dimethyl sulfoxide) and monensin (1 μM in ethanol). Immediately before each of the additions and 4 min after the last, aliquots of 1 × 10^6 cells were removed and centrifuged. The $^{45}Ca^{2+}$ recovered from the media and the pellets was assayed in a Beckman beta counter (for further details, see Fasolato et al., 1991).

Metabolic Labeling, Endoglycosidase H (Endo-H) Digestion, and Velocity Gradient Centrifugation

Subconfluent monolayers in 100-mm dishes were washed (three times) with DMEM and then incubated for 5 h at 37°C with methionine-free medium containing 100 μCi/ml [^{35}S]methionine. All of the subsequent steps were carried out at 4°C. The cells were washed twice with PBS and scraped off with a rubber policeman in 600 μl of lysis buffer, 150 mM NaCl, 15 mM $MgCl_2$, 1 mM EGTA, 50 mM HEPES-KOH, 10% glycerol, 1% Triton X-100, and a mixture of protease inhibitors (1 mg/ml chymostatin, 1 mg/ml leupeptin, 1 mg/ml antipain, 1 mg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5). After incubation for 60 min, the lysates were centrifuged for 10 min in a Microfuge and the supernatants and 20 μl of anti-CSQ Ab were incubated overnight on a rotating device. Immunocomplexes were bound for 1 h to protein A-Sepharose beads and then washed (four times) with the cell lysis buffer (containing, however, 0.2% Triton X 100) and (once) with the Endo-H buffer (75 mM sodium citrate, pH 5.5, 0.1% Triton X-100, 45 mM 2-mercaptoethanol, and protease inhibitors as above). The samples were incubated with or without 5 mU of Endo-H for at least 5 h at 37°C. The reaction was stopped with the SDS-PAGE sample buffer.

To investigate the effects of BFA treatment on the Endo-H resistance of CSQ, the cells were incubated in methionine-free medium with or without BFA (5 μg/ml) for 30 min at 37°C, then pulse-labeled for 10 min with [^{35}S]methionine and chased in DMEM containing 0.1 mM unlabeled methionine, supplemented with 15% fetal calf serum and 100 U/ml penicillin and streptomycin for 2 h. The immunoprecipitates were prepared, incubated in the presence or absence of the Endo-H enzyme, and processed as described above. For the CSQ turnover experiments, cell monolayers were incubated with methionine-free medium containing 100 μCi/ml [^{35}S]methionine for 30 min and then chased for times from 0 to 48 h.

For CSQ gradient analysis, A2 cells and a low-speed (3000 × g for 10 min) pellet isolated from a postnuclear supernatant were lysed (1 h, 4°C) with 1% Triton X-100 in the Ca^{2+}-free lysis buffer described for metabolic labeling. Microsomes isolated from the rat quadriceps muscle and an authentic chicken CSQ standard were similarly treated in parallel. These preparations were then cleared by centrifugation at 12,000 × g for 10 min and the pellet was resuspended with the same volume of 1% Triton X-100, 0.5% sodium deoxycholate, and 2% SDS in the same buffer (1 h, 18°C). After recentrifugation as above, the two series of supernatants were loaded onto 4.5-ml linear 5–20% sucrose gradients prepared in lysis buffer containing 0.5% Triton X-100. After centrifugation for 16 h at 45,000 rpm in a SW50.1 rotor (Beckman Instruments, Palo Alto, CA), fractions of 0.4 ml were collected. Samples from lysates and gradient fractions were analyzed by SDS-PAGE (8% polyacrylamide gels) according to the method of Laemmli (1970). The Western blotting procedures were as described (Raichman *et al.*, 1995). For autoradiography, the gels were first fixed in methanol:water:acetic acid (40:50:10), then soaked in Amplify (Amersham), dried, and exposed to Hyperfilm-MP (Amersham) at room temperature.

RESULTS

Nature of CSQ-Rich Vacuoles in Stably Infected L6 Myoblasts

The nature of the membrane-bound dense vacuoles rich in CSQ present in A2, growing L6 myoblasts stably overexpressing the Ca^{2+}-binding protein, previously described by Raichman *et al.* (1995; see also Figure 1, B and D–F), was further investigated by combining biochemistry and immunocytochemistry. To establish whether their CSQ, a glycoprotein with a single oligosaccharide chain, had traveled across the GC, experiments were carried out with Endo-H. The small amounts of CSQ recovered from the incubation medium bathing the infected cells, i.e., the fraction most likely released by constitutive secretion after escape from the ER, were found to be resistant to the enzyme as expected for a GC-processed glycoprotein (Figure 1Ab). In contrast, in the CSQ from cell lysates, the enzyme treatment induced a distinct migration increase; i.e., the protein was Endo-H sensitive (Figure 1Aa). Because in these cells, only a small part of the CSQ is located within the ER cisternae and most of it is within dense vacuoles (Raichman *et al.*, 1995; and see below), this result strongly suggests the protein enclosed within the latter structures has never trafficked through the GC. Support to this conclusion came from high-

resolution immunocytochemical studies. Of four ER markers coinvestigated with CSQ by dual immunogold labeling of ultrathin cryosections, only PDI and BiP were not detected within the typical vacuoles (Figure 1B; our unpublished results; Raichman *et al.*, 1995). In contrast, clear immunogold signals within dense vacuoles were obtained with the two other ER markers, CRT (Figure 1D) and the membrane protein CNX (Figure 1E). CRT-addressed gold particles (Figure 1D) appeared distributed throughout the lumen of the vacuoles, intermixed with those of CSQ (average CRT:CSQ immunogold particle ratio in 20 randomly selected vacuoles = 1:102), and CNX particles appeared more concentrated near the surface (Figure 1E), as expected for a membrane protein known to protrude within the lumen.

The occurrence of ER lumenal components within CSQ-rich dense vacuoles could be due to their intermixing with the overexpressed protein during formation of the organelles. Alternatively, they could be the result of a general alteration in the molecular organization of the ER taking place in response to CSQ overexpression, with an ensuing increased expression and misdistribution of other components. To investigate this problem, the levels of various ER proteins were established by Western blotting. As previously reported by Raichman *et al.* (1995), no trace of CSQ was observed in control C1 cells, but in the A2 overexpressor clone primarily used in the present study, CSQ appeared as a major band of ~52 kDa, expressed at levels in the range of skeletal muscle, accompanied by a minor (5–10%) faster running band. In another overexpressor clone, A3, the faster band predominated, and authentic CSQ was comparatively scarce (see Raichman *et al.*, 1995). Similar levels of expression in control and CSQ overexpressing cells, reported by Raichman *et al.* (1995) for PDI, the SERCA Ca^{2+} pump, and the IP$_3$ receptor, were confirmed in the present study and extended to three additional ER markers, BiP, CRT, and CNX, and to the lysosomal enzyme CD (Figure 2).

Additional information about the nature of the dense vacuoles was obtained by experiments with GC and lysosome markers. Figure 3, A and B, shows results of L6 cells of the A2 clone labeled by immunofluorescence for CSQ and either DAMP or lgp120; i.e., the weak amine used to reveal lumenal acidity and a membrane protein specific for lysosomes and late endosomes. As can be seen, with these markers only some CSQ-rich vacuoles appeared positive, and many others remained clearly negative. Figure 3, C–E, illustrate the effects of BFA, the drug that blocks the traffic from the ER to the GC and thus induces the intermixing of the two endomembrane systems. Before drug treatment, the GC marker mannosidase II is shown by immunofluorescence to
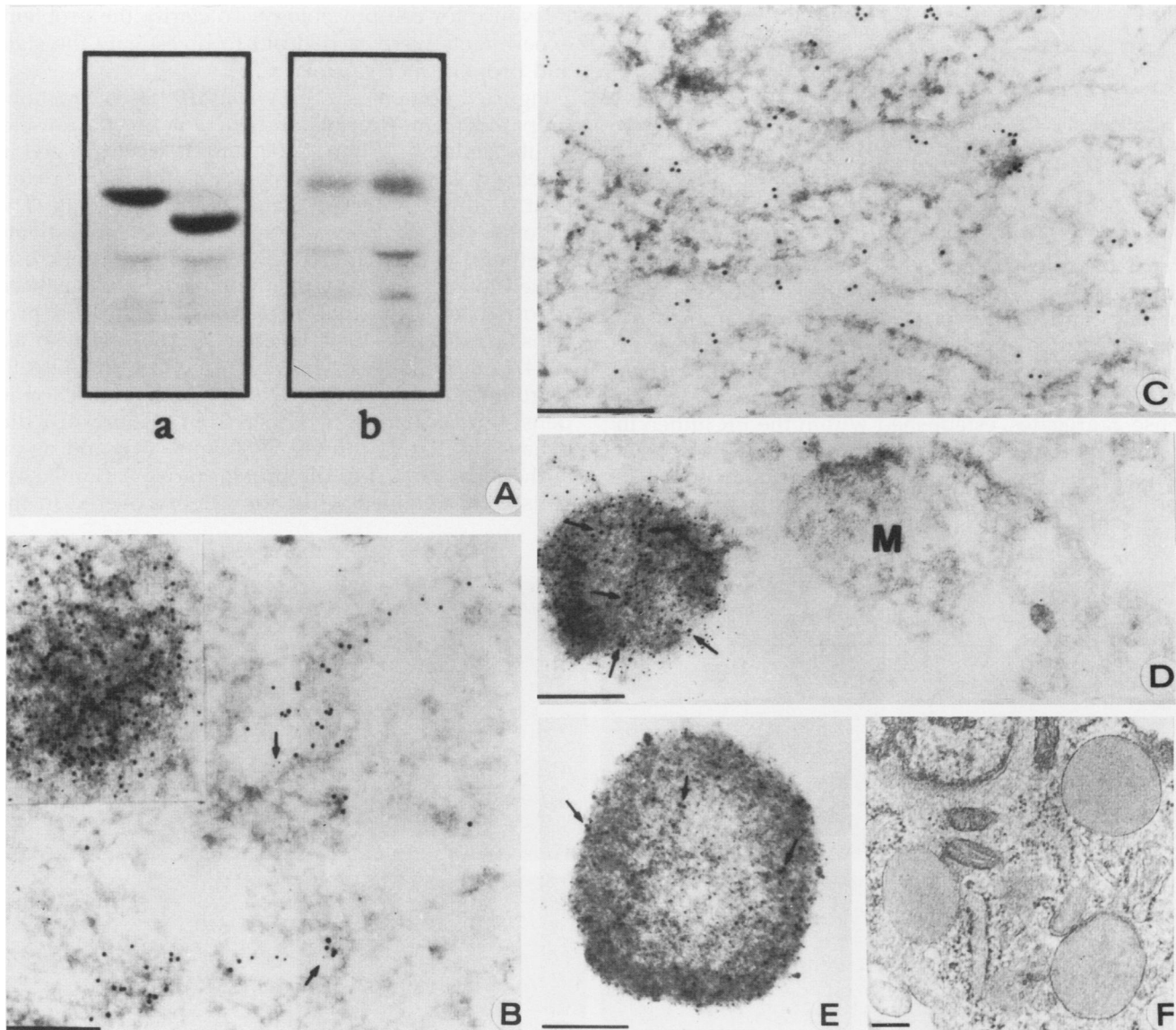
Figure 1. A2 clone cells: Endo-H sensitivity and ultrathin cryosection immunogold labeling of CSQ and ER markers. (A) SDS-PAGE and ^{35}S autoradiography of CSQ immunoprecipitated from cells (a) and the incubation medium (b) before (left) and after (right) treatment with Endo-H. Representative of the results from three consistent experiments. (B) CSQ within the ER cisternal lumen (small gold particles) and PDI (large gold particles). (Inset) Dense vacuole rich in CSQ (large gold particles) where no signal for PDI (small gold particles) was detected. Large gold particles reveal CRT labeling of the ER cisternal lumen (C) and of a dense vacuole (D, arrows) rich in CSQ as revealed by small gold particles. M, mitochondrion. (E) CNX labeling (large gold particles, arrows) of another dense vacuole rich of CSQ labeled by small gold particles. (F) Conventional electron microscopy of a cytoplasmic area including three vacuoles: note their dense homogeneous content closely apposed to the limiting membrane. Bars: B–F, 0.25 μm.

exhibit in A2 cells its typical discrete perinuclear distribution (Figure 3C), and 30 min after BFA, the enzyme showed a diffuse distribution throughout the cytoplasm (Figure 3D), largely overlapping that of ER markers. In spite of this ER–GC intermixing, however, the vacuolar CSQ immunolabeling pattern remained unchanged (Figure 3E). Moreover, the Ca^{2+}-binding protein of the BFA-treated cells re-

mained Endo-H sensitive, i.e., unchanged with respect to untreated cells (compare Figure 3F to Figure 1Aa). These results strongly suggest that, in the conditions of the ER–GC intermixing experiment, the dense CSQ-rich vacuoles of A2 cells (or at least their lumenal CSQ aggregates) remain largely discrete. As a consequence, oligosaccharide chain processing of their CSQ molecules cannot take place.
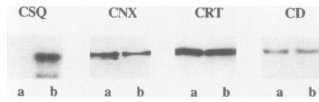
What Is the State of CSQ within Dense Vacuoles?

The experiments reported so far demonstrate that, although apparently discrete, the dense CSQ-rich vacuoles and the ER of A2 cells share a number of typical features and the vacuoles could, therefore, be considered specialized subdomains of the ER. Their cellular significance is, however, not clear. Various dense aggregates, established within the ER lumen in a variety of cell types as a consequence of protein overexpression, both spontaneous and after transfection, have in fact been considered artifacts, with no

relevance for cell physiology. To clarify the problem, experiments were carried out to investigate the state and properties of vacuolar CSQ.

Figure 4 compares CSQ Western blots of fractions prepared from A2 cells and rat quadriceps muscle, solubilized with Triton X-100, and then centrifuged in a velocity sucrose gradient containing the same detergent, without both Ca^{2+} and reducing agents. The rationale of this experiment was to investigate whether the aggregated CSQ molecules are cross-linked to each other by disulfide bridges as has been reported for artifactual aggregates of other ER proteins (Tooze *et al.*, 1989; Valetti *et al.*, 1991; Field *et al.*, 1994). As can be seen, however, the CSQ of A2 cells (Figure 4A) and of a subcellular fraction enriched in dense granules obtained therefrom (Figure 4B) did behave as that of muscle SR (Figure 4C) and as an authentic standard of the protein (arrow). Similar results were obtained when the CSQ recovered in the
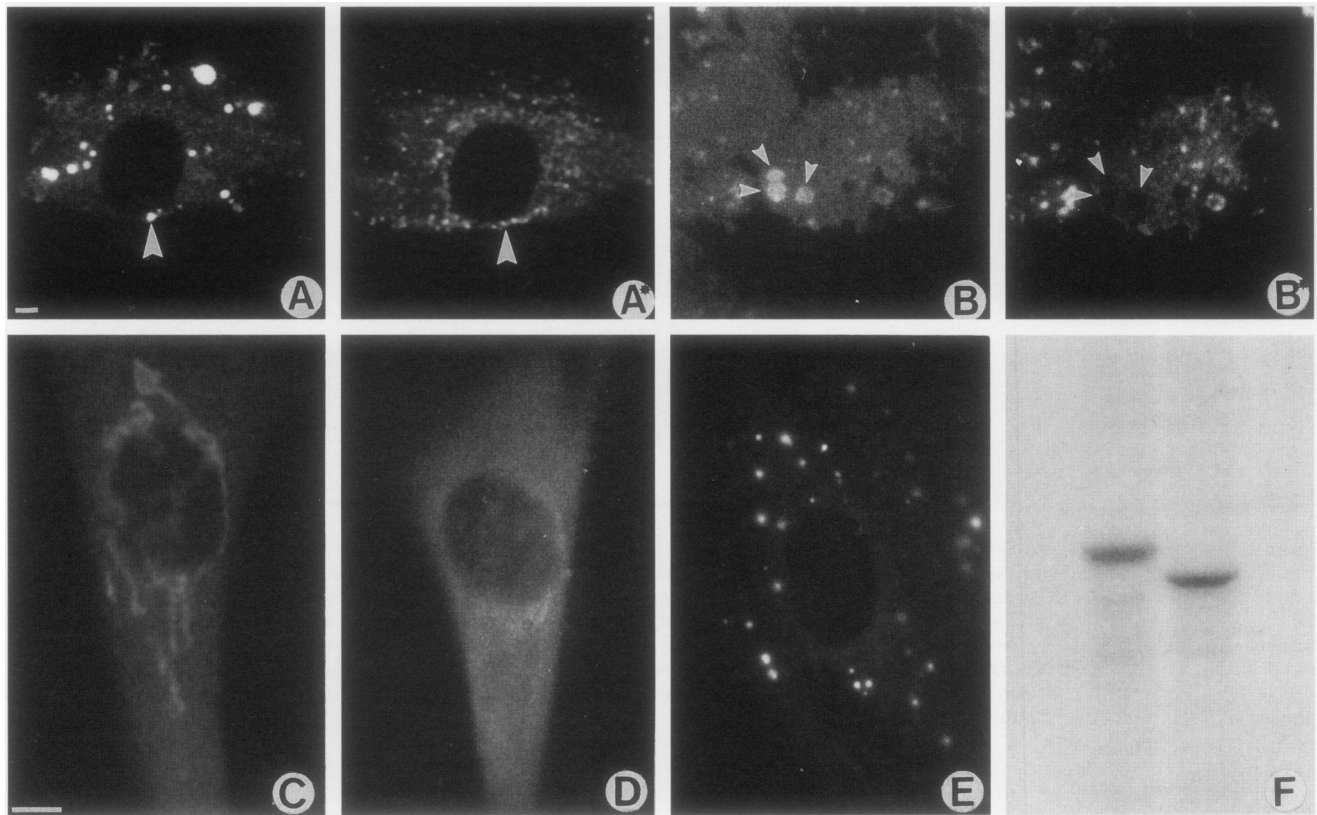

Figure 3. CSQ-rich vacuoles of A2 clone cells: dual confocal immunolabeling with DAMP and lgp120 and effects of cell treatment with BFA. (A, A*, B, and B*) Dual confocal labeling results with CSQ (A and B) and DAMP (A*) and lgp120 (B*). The arrowheads in A point to a vacuole positive for both CSQ and DAMP and those in B point to three large vacuoles positive for CSQ and negative for lgp120. (C and D) Effects of cell treatment with BFA, revealed by immunolabeling for mannosidase II. In the untreated cell (C), the distribution of the enzyme is GC-type, around the nucleus; after BFA (D), it becomes ER-type, distributed throughout the cytoplasm. (E) Distribution of CSQ in two BFA-treated cells is still in discrete vacuoles; i.e., it appears unaffected by BFA. Bars: A (valid also for B) and C (valid also for D and E), 2 μm. (F) Effect of Endo-H on the SDS-PAGE migration of metabolically labeled CSQ immunoprecipitated from cells treated with BFA: left lane, before; right lane, after treatment with the enzyme. Representative of the results in two consistent experiments.

Figure 4. CSQ gradient analysis of Triton X-100 lysates from A2 cells (A), a dense vacuole-rich subcellular fraction isolated therefrom (B), and microsomes isolated from the rat quadriceps muscle (C). Western blots of velocity gradient fractions are shown. Lysates were prepared and centrifuged in the presence of Triton X-100, without Ca^{2+} and reducing agents. Loading was with either 500 (A and C) or 50 (B) μg of protein. The arrow at the top indicates the peak position of the authentic CSQ standard run in parallel. Representative of four (A) and two (B and C) consistent experiments.
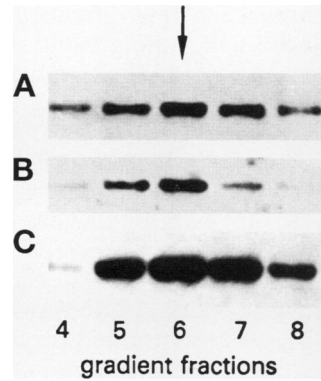
pellets of the first Triton X-100 extractions (on the average ~30% of the total) was solubilized with a detergent mixture (Triton X-100, sodium deoxycholate, and SDS) and centrifuged in gradients such as those of Figure 4. Thus, these results exclude the existence of any cross-linking among the CSQ molecules of the granule content.

The following series of experiments investigated the interactions of the CSQ aggregates with overexpressed CRT, a chaperone of the ER (and SR) lumen that resembles CSQ in both its general structure and Ca^{2+}-binding properties (Michalak *et al.*, 1992). The L6 cells employed were from the A2 and C1 clones, in both cases transfected according to a transient protocol with a tagged CRT cDNA and analyzed 40 h later. Figure 5 shows the results. As can be seen, overexpressed CRT remained diffusely distributed in the ER and failed to concentrate in the dense vacuoles with CSQ, independently of whether in A2 cells or in C1 cells cotransfected with both proteins (Figure 5, A and B).

Information about two additional properties of the infected protein came from biochemical experiments. When L6 cells of the A2 CSQ-overexpressor clone were first pulse-labeled for 30 min with [^{35}S]methionine and then chased in the conventional medium for up to 30 h, the *t* of the Ca^{2+}-binding protein, purified by immunoprecipitation followed by SDS-PAGE, was found to be ~12 h, half the value recorded in rat primary myotubes (24 h, Zubrzycka and MacLennan, 1976). Furthermore, the role of dense vacuoles in Ca^{2+} homeostasis was investigated by experiments in which we assayed the radioactivity released to the Ca^{2+}-free medium from C1 and A2 cells first loaded at the equilibrium (48 h) with ^{45}Ca and then treated in sequence (with no intervening washes) 1) with the receptor stimulant AVP, administered with TG, the blocker of SERCA; 2) with the Ca^{2+} ionophore IONO; and 3) with the H^+ ionophore monensin. These treat-
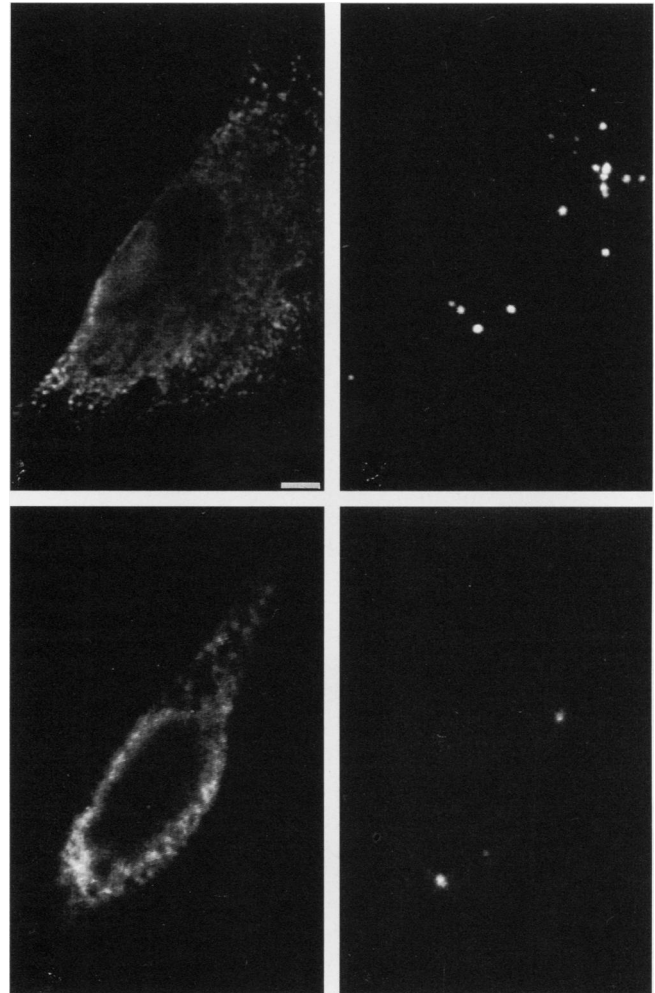

Figure 5. Dual confocal immunofluorescence of exogenous CRT and CSQ in L6 cells. (A and A*) A2 cells transiently transfected for HA1-tagged CRT. (B and B*) C1 cells simultaneously cotransfected for both CSQ and CRT. Fixation was made 40 h after transfection. (A and B) CRT. (A* and B*) CSQ. Bar: A (valid also for B), 2 μm. Representative of four consistent experiments.

ments are expected to induce selective release of Ca^{2+} 1) from the ER stores expressing the IP_3 receptor and loaded by the ER-specific pump, 2) from any additional store excluding those with acidic content, and 3) from any store with acidic content. As can be seen in Figure 6, the largest difference emerged in the Ca^{2+} pool released by IONO. Compared with C1 cells, release from A2 overexpressor cells induced by the Ca^{2+} ionophore was in fact increased by about 250%. In contrast, with AVP-TG release, the difference was small, but with monensin, it was +65%. We conclude that CSQ segregated within dense vacuoles does play a role in the Ca^{2+} storage of stably overexpressing L6 myoblasts even if uptake and release of the cation appear to be carried out by mechanisms different from those typical of the rest of the ER.
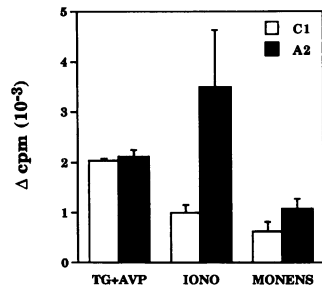
Figure 6. Release of ^{45}Ca from L6 cells, C1 (open bars) and A2 (solid bars). Cells were prelabeled at the equilibrium (48 h) with the tracer and then exposed in sequence to the indicated treatments (4 min each).

Generation and Turnover of CSQ-Rich Vacuoles

In the preceding experiments, the L6 cells used were from the stably overexpressor A2 clone or from the C1 clone transfected for relatively long periods of time (40 h) with the chicken CSQ cDNA. In these cells the overall picture was dominated by already existing dense vacuoles rich of CSQ, and no clear images suggestive for their formation could be identified in the ER by electron microscopic immunocytochemistry. To obtain information about the latter process, we turned to short-term transient transfections of control C1 cells. Together with the plasmid containing the cDNA of CSQ the cells received that of β-galactosidase, used to help in the identification of transfectants.

In a first group of experiments, confocal microscope triple immunofluorescence analyses were carried out to reveal, in addition to β-galactosidase and CSQ, DAMP. The aim was to reveal the kinetics of not only the appearance but also the acidification of dense vacuoles. Up to 13 h after cell transfection, no CSQ was revealed by immunofluorescence. One hour later the Ca^{2+}-binding protein appeared in β-galactosidase-positive cells, arranged in a diffuse network similar to the pattern of typical ER markers such as CRT and PDI (see Raichman *et al.*, 1995). At this time only few typical dense vacuoles were visible (Figure 7A). At 15 h (Figure 7B), the CSQ-rich vacuoles were increased in number, however, remaining clearly below the complement of the stable A2 overexpressor, and were always negative for DAMP (Figure 7, compare B and B*). At the following times investigated (20 and 44 h), the dense vacuoles appeared numerous in almost all CSQ-positive cells. Colabeling for DAMP, however, was limited to only some cells; the others were apparently nonacidic in their lumen (Figure 7, C, C*, D, and D*).

On the basis of the above confocal microscopy results, the 14- and 20-h points were chosen to investigate by immunoelectron microscopy the initial and intermediate steps of vacuole formation, respectively. Because we needed to scrutinize large cytoplasmic areas of the few (~10%) transiently transfected CSQ-labeled cells, we turned to preparations embedded in the water-soluble acrylic resin LR white. Figure 8 shows that, under these conditions, the level of CSQ

immunolabeling remained comparable to that of cryosections and the general ultrastructure was well preserved, although membranes appeared electron-lucent. This was expected because cells had not been exposed to OsO_4.

Among 14-h–transfected cells, CSQ immunolabeling was highly variable, from low to high levels, distributed throughout the ER cisternae. At discrete sites, however, gold particle clusters were apparent, labeling dense aggregates occupying only part of the lumen or entire cul-de-sac. Dense vacuoles, apparently discrete and of round profile, were still rare yet visible in at least a few of these cells (Figure 8A). At 20 h, the ER labeling was strongly reduced in most ER cisternae except for those in direct continuity with expansions containing CSQ dense aggregates, which sometimes appeared to be moth eaten (Figure 8B). Typical heavily labeled vacuoles were frequent at this time, whereas other structures, including the GC, were completely unlabeled (Figure 8C). Thus, the confocal and ultrastructural images strongly suggest that the formation of vacuoles was due to the progressive aggregation of CSQ molecules, initiated within the lumen of ER cisternae.

The final problem we investigated was whether, and to what extent, the segregated content of established dense vacuoles could be reached by the newly synthesized Ca^{2+}-binding protein. For these studies cells from the C1 and A2 clones were transfected with the cDNA of the rabbit Ca^{2+}-binding protein including in its sequence the molecular HA1 tag. In transfected C1 cells, CSQ immunolabeling and dense vacuoles appeared at 14 and 15 h, respectively, according to the schedule previously defined with the chicken protein (see above and Figure 7). Also in A2 cells, stably overexpressing the chicken CSQ and thus exhibiting dense vacuoles from the beginning, the transfected tag signal remained inappreciable until 13 h after transfection. At 14 h a diffuse ER tag labeling appeared, however, without any clearly visible CSQ-rich vacuoles (Figure 9A). One hour later the number of the dense vacuoles was not significantly changed (11.3 ± 1.1 and 11.8 ± 0.8 dense vacuoles/cell, average ± SEM; n = 20 cells for each point). Changed however was the distribution of the tag, with strong reduction of the diffuse labeling and definite labeling of most dense vacuoles (332 of 412 counted). Within these vacuoles the distribution of the tag was homogeneous, apparently intermixed with the nontagged CSQ. At later times, i.e., up to 40 h, the number of dense vacuoles was progressively increased, and almost all of them were positive for the tag. When we take into account the relatively slow turnover of CSQ, the appearance within an hour (14–15 h from transfection) of the tag in most dense granules without significant increase of that population excludes the possibility that the process is due only to new organelle assem-
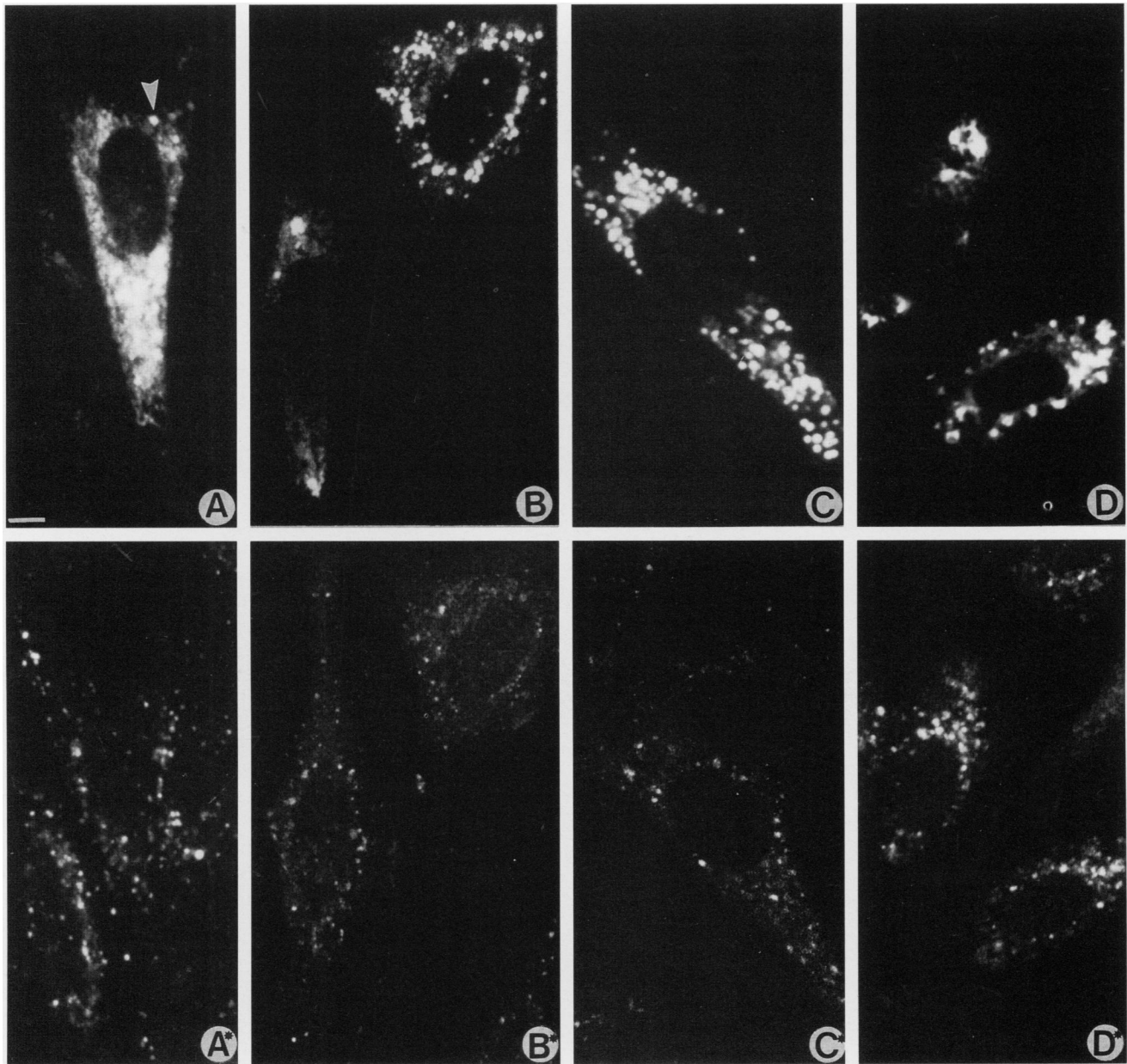
Figure 7. Dual confocal immunofluorescence of CSQ (A–D) and DAMP (A*–D*) in C1 cells transiently transfected for the protein and analyzed after 14 (A), 15 (B), 20 (C), and 44 (D) h. Bar: A (valid for all panels), 2 μm.

bly. We conclude therefore that most preassembled dense vacuoles, although apparently discrete, are able to promptly receive the newly synthesized CSQ from the ER, with continuous remodeling of their content.

DISCUSSION

Among the Ca^{2+}-binding proteins segregated within the ER lumen, the state of CSQ appears unique. The protein differs in fact from others, such as CRT, be-

cause it does not include the C-terminal KDEL tetrapeptide and, therefore, is excluded from the specific shuttling system to maintain its ER segregation and because it is not distributed evenly throughout the ER but is concentrated into dense discrete aggregates that in striated muscle fibers are located at physiologically critical sites, i.e., within SR terminal (and corbular) cisternae. The previous results by Raichman *et al.* (1995) have shown that in L6 and other cell types overexpression of CSQ leads to the formation of dense
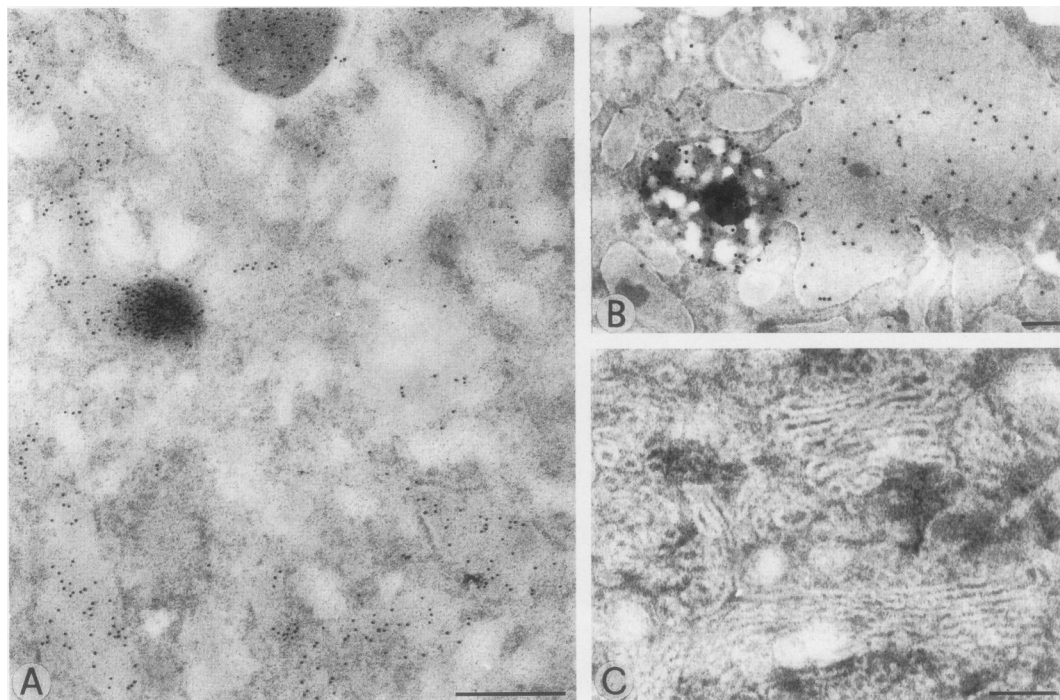
Figure 8. CSQ immunogold labeling of LR white-embedded C1 cells transiently transfected with the corresponding cDNA 14 (A) and 20 (B and C) h before fixation. (A) CSQ-positive cell (at 14 h) shows labeling within the ER, with a dense intralumenal aggregate (center) as well as a larger body (top) also positive for CSQ. The latter most probably corresponds to a small dense vacuole. (B) The field (at 20 h) is largely occupied by a swollen moderately labeled ER cisterna, continuous with a cul-de-sac occupied by a comb-like aggregate of CSQ. (C) GC at 20 h from transfection, completely free of CSQ labeling. Bars, 2 μm.

vacuoles filled with the Ca^{2+}-binding proteins, at that time identified as post-GC–prelysosomal organelles. Herein we demonstrate in contrast that the dense vacuoles develop as distinct ER subdomains and that various properties of their CSQ lumenal content might be valid also for the SR and ER of the cells where the Ca^{2+}-binding protein is physiologically expressed.

Nature and Formation of Vacuoles

As far as the cytological nature of dense vacuoles, our results in stably overexpressor L6 cells indicate that they consist of specialized ER subdomains. This conclusion is based on the results of experiments that had not been carried out by Raichman *et al.* (1995): the Endo-H sensitivity of the CSQ oligosaccharide chain, a property that excludes trafficking of the vacuolar protein through the GC, and the demonstration that the organelle contained two bona fide ER markers, CRT and CNX. The fact that two additional markers, BiP and PDI, could not be detected (see also Raichman *et al.*, 1995) is of interest because it does mimic the situation previously observed within the muscle SR terminal cisternae where BiP, excluded from the dense CSQ aggregates, was detected only in the gap between the latter and the limiting membrane, whereas CRT and CNX were enriched in the terminal with respect to longitudinal cisternae (Volpe *et al.*, 1992).

Further support for the ER nature of CSQ-rich dense vacuoles comes from the evidence about their

formation obtained by transient transfection experiments. Appearance of these organelles was shown in fact to take place after an initial lag period during which the exogenous protein, largely distributed throughout the ER lumen, began to aggregate at discrete sites. Formation of CSQ aggregates in L6, probably the mechanism by which transport of the protein to the GC and ensuing secretion are largely prevented, resembles events occurring during the development of skeletal muscle fibers. Also in that system, in fact, dense CSQ aggregates, which first appear irregularly distributed throughout the ER/SR, reach their exclusive localization within the SR terminal cisternae only a few days later (Flucher, 1992; Villa *et al.*, 1993a). Once established, however, the similarity of vacuoles with muscle cisternae becomes less stringent, because the first appear as discrete organelles, whereas the others maintain their continuity with the rest of the ER. The state of dense vacuoles, however, appears more complex than shown by conventional electron microscopy. The lack of processing of their oligosaccharide chain, from Endo-H sensitive to resistant, even when the cells were treated with BFA, suggested the stable separation of these organelles or at least the lack of mixing of their CSQ aggregates with the other lumenal ER and GC components. This, however, appears not to be the case with CSQ itself, as revealed by the rapid intermixing established within individual vacuoles of A2 clone cells by the two CSQs, the chicken form stably overexpressed
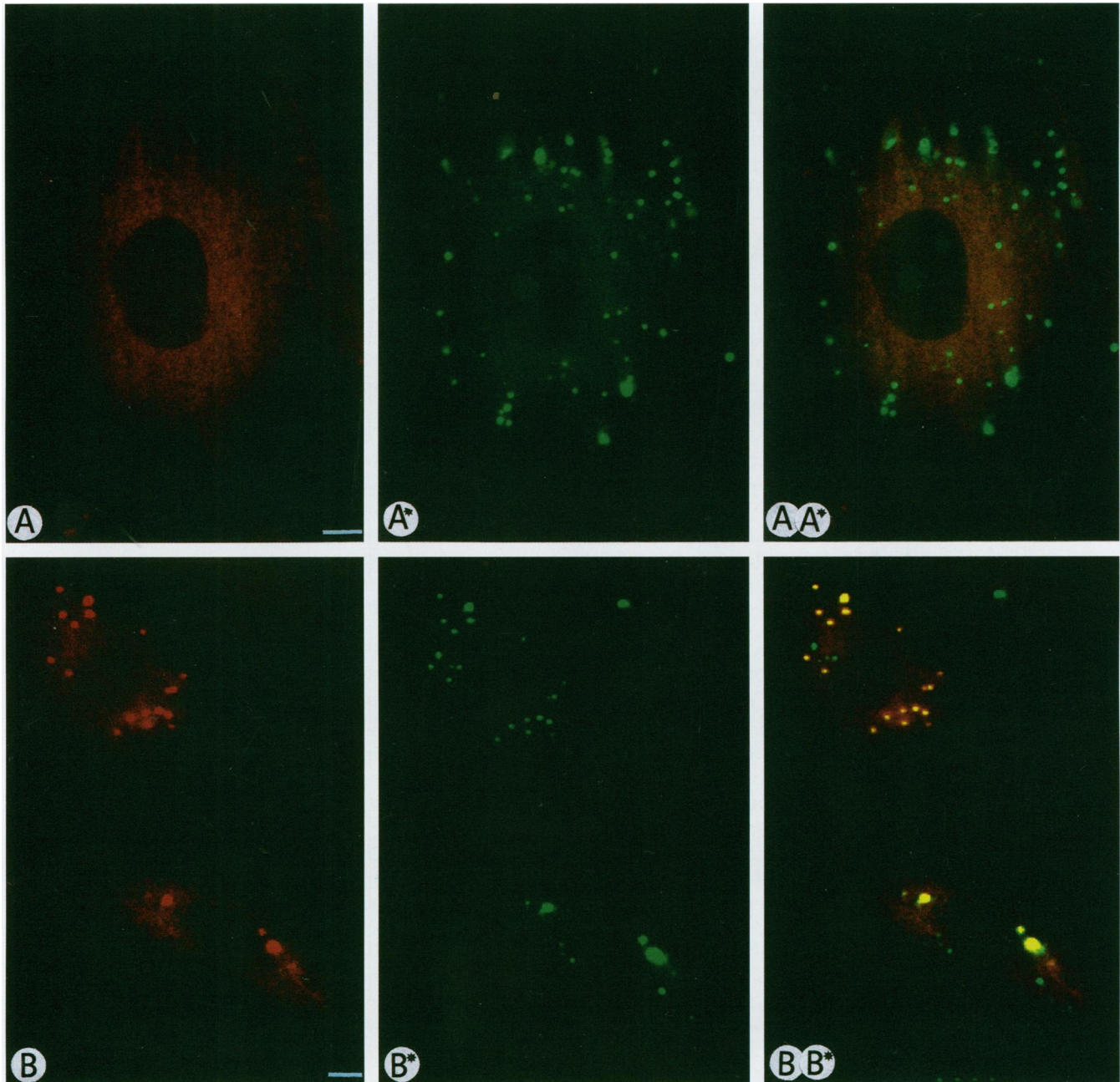
Figure 9. Dual CSQ confocal immunolabeling in A2 cells transiently transfected for the HA1-tagged form of the protein 14 (A), and 15 (B) h before fixation. (A and B) Tag signal. (A* and B*) Total CSQ in the same cells. (AA* and BB*) Superimpositions of A with A* and B with B*. At 14 h, the transfected tagged CSQ is still distributed throughout the ER, with no accumulation within the vacuoles containing the infected protein, whereas by 15 h most of the tag signal has moved to the vacuoles, almost all of which appear, thus, doubly labeled (yellow color in BB*). Bars: A and B, 2 μm.

and the tagged form transiently transfected. From these data it appears that dense vacuoles do establish dynamic connections (possibly by fusion) with the rest of the ER and that this is the mechanism by which an equilibrium of the segregated CSQ is maintained.

Significance and Properties of the CSQ-rich Vacuoles

On the basis of the properties discussed so far, the CSQ-rich vacuoles appear to resemble specific structures rather than artifactual bodies generated by pre-

cipitation of the overexpressed protein. The latter possibility, however, had to be considered because of examples reported to appear in a variety of cell types, not only after induced protein overexpression but also spontaneously. Among the aggregates of the artifactual type, we mention those harboring misfolded proteins bound to, and intermixed with, chaperones, in particular with BiP, including the BiP bodies of yeast (Field *et al.*, 1994; Nishikawa *et al.*, 1994), and the discrete aggregates known as intracisternal granules (pancreas: Tooze *et al.*, 1989) and Russel bodies (B lymphocytes: Valetti *et al.*, 1991) composed of secretory proteins extensively cross-linked by disulfide bridges. Various criteria, however, differentiate CSQ-rich vacuoles from the above structures. First, the experiments carried out by cotransfection of CSQ and CRT (itself a chaperone, and in addition a low-affinity Ca^{2+}-binding protein very similar to CSQ in its general structure and properties; see Michalak *et al.*, 1992) failed to reveal any extensive coaggregation of the two overexpressed proteins, of which only CSQ was mainly concentrated within the vacuoles while CRT remained largely distributed throughout the entire ER. Moreover, other chaperones, BiP and PDI, were completely excluded from the CSQ-rich vacuoles, as was discussed in the preceding section. Second, the treatment with Triton X-100 in Ca^{2+}-free medium, carried out without addition of any reducing agent, induced ample solubilization of L6 cell vacuoles similar in this respect to muscle SR terminal cisternae (Costello *et al.*, 1986; Volpe *et al.*, 1992). The parallel gradient centrifugation of preparations solubilized by Triton X-100 and by a detergent mixture (Triton X-100, sodium deoxycholate, SDS) from both L6 and muscle lead to the distribution of CSQ into the same few fractions where the authentic protein standard was also recovered. Thus, within vacuoles—as it is known for SR terminal cisternae, and at variance with pancreatic intracisternal granules, Russel and other bodies (Tooze *et al.*, 1989; Valetti *et al.*, 1991; Field *et al.*, 1994; Nishikawa *et al.*, 1994)—the segregated CSQ molecules are not cross-linked by disulfide bridges. Finally, overexpression and aggregation of CSQ in L6 vacuoles did not result in a stress to the cell. In fact, chaperones, in particular BiP, failed to be overexpressed in these L6 cells (our unpublished observations; see also Raichman *et al.*, 1995) but were overexpressed in cells bearing the artifactually aggregate structures discussed above (Tooze *et al.*, 1989; Nishikawa *et al.*, 1994).

The results with ^{45}Ca are also in apparent agreement with the nonartifactual nature of vacuoles. In our previous studies, we reported that in growing CSQ-overexpressing L6 cells, the IP_3-mediated intracellular Ca^{2+} concentration responses were unchanged, a conclusion consistent with the present demonstration that the AVP-TG–sensitive Ca^{2+} pool is not significantly different in C1 and A2 cells labeled at equilibrium with

^{45}Ca. Another pool, however, insensitive to AVP and TG and released by the Ca^{2+} ionophore IONO was more than tripled in A2 cells. Because all other investigated proteins known to participate in Ca^{2+} homeostasis were found to be unchanged in the A2 cells, the simplest explanation for the enlargement of the IONO pool is that CSQ binding of the cation takes place even when the protein is segregated within the dense vacuoles. On the basis of the latter assumption and on the expression level of the protein in the A2 clone, the Ca^{2+}:CSQ molar ratio at rest can be calculated to be ~25:1, i.e., about half the B_{max} of the protein. This suggests the concentration of free Ca^{2+} within the vacuoles to be around the K_d of CSQ, i.e., 1 mM, similar to the value known to exist within the ER of other cell types (Bastianutto *et al.*, 1995; Montero *et al.*, 1995). As far as the mechanisms of Ca^{2+} uptake into and release from the vacuoles, our present evidence only excludes a short-term (minute) involvement of both SERCAs and IP_3 receptors, i.e., the mechanisms predominant in the ER. Because, however, multiple mechanisms are known to operate in that and other endomembrane systems (Pozzan *et al.*, 1994; Waldron *et al.*, 1995; Pizzo *et al.*, 1997), the degree of specificity of the vacuole Ca^{2+} transport cannot be established yet.

Destiny of Vacuoles

Pulse-labeling experiments with [^{35}S]methionine revealed CSQ to undergo degradation with *t* of ~12 h, a value only twice as fast as that reported for the endogeneous CSQ in rat primary myotubes (Zubrzycka and MacLennan, 1976). Our previous studies (Raichman *et al.*, 1995), carried out primarily in the A3 clone, had shown many vacuoles to be acidic and positive for CD immunocytochemistry. In A2, the overexpressor clone most extensively investigated herein, we also observed DAMP (acidity), CD, and also lgp120, a lysosomal membrane protein. Thus these data suggest some involvement of lysosomes that is variable, however, between the two clones. In view of the fact that acidic vacuoles, when analyzed by conventional electron microscopy, exhibit only few membranes and no organelles within their content, the most likely process involved in their formation appears ER-lysosome diversion (Noda and Farquhar, 1992) rather than crinophagy (Smith and Farquhar, 1966) or autophagocytosis (Dunn, 1990). Also for their degradation mechanism, the CSQ-rich vacuoles appear to be distinct from the ER lumenal protein bodies discussed previously. In fact, misfolded protein-chaperone aggregates are known to turn over rapidly (Amara *et al.*, 1989; Bonifacino *et al.*, 1989; Field *et al.*, 1994; Kuznetsov *et al.*, 1996; Willnow *et al.*, 1996) by nonlysosomal mechanism(s), whereas pancreatic intracisternal granules are eliminated by autophagocytosis, as

revealed by the heterogeneous content of the resulting vacuoles (Tooze et al., 1990).

Conclusions

The results that we have reported clarify various aspects of the intracellular life of CSQ when expressed by L6 myoblasts. The aggregation of the newly synthesized protein within the ER lumen closely resembles the process occurring within the SR of striated muscle fibers and appears due to a specific property of CSQ that CRT, the other Ca^{2+}-binding protein, is unable to share even when cooverexpressed at high levels. Most likely it is this aggregation that induces retention of CSQ within the ER, by precluding the entrance of the protein into transport vesicles. In the subsequent step, CSQ aggregates are converted into specialized ER subdomains, a population of dense vacuoles that appear most often distinct but are in a probably transient dynamic continuity with the rest of the ER. Elimination of these vacuoles appears to take place slowly (t of CSQ = 12 h), at least in part by lysosomal diversion. Thus these results identify L6 as a cell model in which various cellular and molecular mechanisms of the CSQ intracellular life could be further investigated. Knowledge about these mechanisms might be of importance also for the understanding of processes by which the complexity of the SR in striated muscle fibers is ultimately established.

ACKNOWLEDGMENTS

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