Head-to-tail oligomerization of calsequestrin: a novel mechanism for heterogeneous distribution of endoplasmic reticulum luminal proteins

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Many proteins retained within the endo/sarcoplasmic reticulum (ER/SR) lumen express the COOH-terminal tetrapeptide KDEL, by which they continuously recycle from the Golgi complex; however, others do not express the KDEL retrieval signal. Among the latter is calsequestrin (CSQ), the major Ca$^{2+}$/H$^{+}$-binding protein condensed within both the terminal cisternae of striated muscle SR and the ER vacuolar domains of some neurons and smooth muscles. To reveal the mechanisms of condensation and establish whether it also accounts for ER/SR retention of CSQ, we generated a variety of constructs: chimeras with another similar protein, calreticulin (CRT); mutants truncated of COOH- or NH$_2$-terminal domains; and other mutants deleted or point mutated at strategic sites. By transfection in L6 myoblasts and HeLa cells we show here that CSQ condensation in ER-derived vacuoles requires two amino acid sequences, one at the NH$_2$ terminus, the other near the COOH terminus. Experiments with a green fluorescent protein GFP/CSQ chimera demonstrate that the CSQ-rich vacuoles are long-lived organelles, unaffected by Ca$^{2+}$ depletion, whose almost complete lack of movement may depend on a direct interaction with the ER. CSQ retention within the ER can be dissociated from condensation, the first identified process by which ER luminal proteins assume a heterogeneous distribution. A model is proposed to explain this new process, that might also be valid for other luminal proteins.

Introduction

The lumen of the ER is now recognized as a key compartment of the cell in which multiple functions are carried out and/or regulated by resident, nonmembrane proteins (for reviews see Meldolesi and Pozzan, 1998; Corbett and Michalak, 2000; Molinari and Helenius, 2000). The majority of the ER luminal proteins terminate with the KDEL amino acid sequence (Pelham et al., 1988). Binding of this signal to specific KDEL membrane receptors at the level of the Golgi complex (Lewis and Pelham, 1990), followed by vesicle recycling, provides a dynamic retrieval mechanism for accumulation of ER luminal proteins. In addition, KDEL-dependent retrieval results in intermixing of the recycling-competent proteins, and therefore may contribute to their diffuse distribution throughout the entire ER system (for reviews see Lewis and Pelham, 1996; Meldolesi and Pozzan, 1998).

However, several proteins that reside in the ER lumen do not terminate with the KDEL retrieval signal. The best known example of a luminal protein without a KDEL signal is calsequestrin (CSQ),* a low affinity–high capacity Ca$^{2+}$-binding protein (MacLennan and Wong, 1971). CSQ is found in dense, highly concentrated (up to 1–2 mmol/liter) filamentous matrices segregated within the terminal cisternae of the sarcoplasmic reticulum (SR). The SR is the ER subcompartment highly developed in striated muscle fibers, and characterized by a precisely defined architecture because of its intimate interaction with the plasmalemma T-tubule.

*Abbreviations used in this paper: Ab, antibody; CRT, calreticulin; CSQ, calsequestrin; GFP, green fluorescent protein; HA, hemagglutinin; SR, sarcoplasmic reticulum.
membrane. In contrast, the SR longitudinal cisternal network distributed around muscle myofibrils is almost completely devoid of CSQ (Cala et al., 1990; Jorgensen et al., 1993; Franzini-Armstrong and Jorgensen, 1994; Meldolesi and Pozzan, 1998). The unique distribution of CSQ is of key physiological importance. Due to their proximity to ryanodine receptors (the SR Ca\(^{2+}\) channels), the condensed CSQ matrices contribute to the regulation of Ca\(^{2+}\)/H\(^{+}\) fluxes (Ohkura et al., 1998; Szegedi et al., 1999) and provide the pool of Ca\(^{2+}\) necessary to trigger and sustain muscle contraction (Franzini-Armstrong and Jorgensen, 1994).

Condensation of CSQ to yield dense organelle cores does not take place in the terminal cisternae only, but also exists within the discrete corbular vacuoles of the heart SR, and within ER cisternae and vacuoles in some smooth muscle and neurons (Waytack et al., 1987; Villa et al., 1991, 1993; Volpe et al., 1991). Moreover, the same process of condensation occurs in other cells (L6 myoblasts, PC12 pheochromocytoma, and HeLa epithelial cells) when transfected with a CSQ expression vector (Papazafiri et al., 1994; Raichman et al., 1995; Gatti et al., 1997). This indicates that intraluminal condensation of CSQ is a physiological property that exists in any cell in which the protein is expressed. The condensed organelle cores, which also contain trace amounts of other ER proteins, remain in equilibrium with a soluble pool of protein (Gatti et al., 1997). The molecular mechanisms responsible for condensation and specific redistribution of CSQ in the lumen of ER/SR are not known.

Here we demonstrate that condensation of CSQ is due to oligomerization. As a result the protein, retained within the ER/SR lumen by an independent mechanism, does assume its typical heterogeneous distribution. We also identify two amino acid sequences that are essential for condensation of the protein, one at the NH\(_2\) terminus (residues 1–15, hereafter referred to as site A) and one near the COOH terminus (residues 337–357, hereafter referred to as site B) of CSQ.

**Results**

**CSQ/CRT tail exchange**

To investigate the mechanism of CSQ condensation within the ER/SR lumen, expression vectors pcDNA CSQ/CRT and pcDNA CRT/CSQ were created. These vectors encode chimeras containing the bulk of the NH\(_2\)-terminal region of either CSQ or calreticulin (CRT) (an ER luminal Ca\(^{2+}\)-binding chaperone) in frame with the COOH-terminal region of the other (Fig. 1 A). Although distinct for KDEL expression (in CRT only) and luminal distribution (condensed and diffuse, respectively), CSQ and CRT share a general structural similarity, especially at their COOH-terminal tail. Thus, the general organization of the two chimeras was expected to largely maintain that of native proteins.

In each experiment, preparations of L6 myoblasts not expressing endogenous CSQ were cotransfected with two expression vectors, one encoding a specific chimera (Fig. 1 A), and the other encoding influenza hemagglutinin (HA)-tagged, full-length CSQ or CRT. Similar experiments were carried out with HeLa cells (unpublished data). As expected, we found by immunocytochemistry with anti-HA antibodies that the distribution of HA-CSQ was punctate (Fig. 1 B). In contrast to CSQ, the distribution of HA-tagged CRT was diffuse (Fig. 1, C and D; Gatti et al., 1997). Ultrastructural immunocytochemistry of the HA-tagged CSQ- and CRT-transfected cells confirmed...
that the CSQ puncta are membrane-delimited vacuoles filled with the Ca\(^{2+}\)-binding protein (Fig. 1 F), whereas the diffuse pattern of CRT is due to its distribution throughout the ER cisternae (Fig. 1 E; Gatti et al., 1997). CSQ/CRT (Fig. 1 B and C) and CRT/CSQ (Fig. 1 D) showed a diffuse but not punctate distribution, indistinguishable from the distribution of the full-length CRT. Likewise, at the electron microscope immunocytochemistry level the chimeras appeared distributed throughout the ER (unpublished data).

No accumulation of either chimera was observed at the level of the Golgi complex. We conclude that both the 67 COOH-terminal amino acid sequence residues and another sequence (so far unidentified) contained in the rest of the CSQ molecule are needed to induce condensation.

**Truncation and site-directed mutants**

To identify the specific amino acid sequences important for CSQ condensation, we prepared a panel of expression vectors including full-length CSQ and various NH\(_2\)- and COOH-terminal truncations (Fig. 2 A). L6 myoblasts and HeLa cells were transfected, each with a single expression vector, and the intracellular distribution of the expressed protein was examined by immunocytochemistry. In all cell types investigated, only the full-length CSQ\(^{1–391}\) (Fig. 2, B and F) and the longest COOH terminus deletion mutant, i.e., CSQ\(^{1–350}\) truncated by removal of 41 amino acids (Fig. 2, C and G), exhibited condensation into puncta (i.e., into vacuoles delimited by a single membrane). However, a careful analysis of the puncta obtained with the two CSQ forms revealed differences between them. In particular, in both L6 and HeLa cells transfected with full-length CSQ\(^{1–391}\), the puncta appeared most often compact (Fig. 1 B’ and Fig. 2, B and F) and only a few exhibited an irregular honeycomb or ring structure surrounding clear areas. In contrast, the puncta containing the truncated CSQ\(^{1–350}\) mutant appeared frequently irregular, especially in L6 cells (Fig. 2, C and G). Ultrastructural immunocytochemistry confirmed that irregular puncta are also vacuoles containing CSQ-positive masses, though alternated to clear areas (Fig. 2 R).
The distribution of the other truncation mutants diverged substantially from that of full-length CSQ\textsubscript{1–391}. Whether truncations were at the NH\textsubscript{2}- or COOH termini, when these mutants were expressed in either L6 or HeLa cells they all showed diffuse staining throughout the ER (Fig. 2, D, E, and H–J), a distribution that was also confirmed by electron microscope immunocytochemistry (Fig. 2 K and unpublished data). This distribution was also seen with the truncated mutant CSQ\textsubscript{1–324}, which corresponds to the region of the protein included in the CSQ/CRT chimera (Fig. 2, D and H). Therefore, we conclude that all of these truncated CSQ forms are unable to condensate into vacuoles.

The punctate CSQ\textsubscript{1–350} mutant and the diffuse CSQ\textsubscript{1–324} mutant differ only by the number of amino acids deleted from the COOH-terminal region (41 and 67, respectively; Fig. 2 A). This indicated to us that some information essential for CSQ condensation must reside in residues 325–350. In addition, the results with NH\textsubscript{2}-terminal–truncated mutants indicated that there was another localization in the NH\textsubscript{2}-terminal region of CSQ essential for its condensation. This conclusion was confirmed by results obtained with the shortest COOH-terminal–truncated mutant, CSQ\textsubscript{1–100}, built with an HA sequence at its COOH terminus in order to distinguish it from the other forms used. When the HA-CSQ\textsubscript{1–100} cDNA was transfected not alone (as shown in the legend to Fig. 2 J), but together with a hybrid protein composed of the full-length CSQ\textsubscript{1–391} attached at its COOH-terminus to the green fluorescent protein (GFP/CSQ), the distribution of the two proteins varied depending on their transfection ratio. When the truncated form predominated over GFP/CSQ (5:1), the two immunolabelings codistributed diffusely through the ER, with no appearance of distinct puncta (Fig. 2, L and M); at lower ratios (1:5) puncta were evident and clearly positive, not only for GFP but also for HA (Fig. 2, N and O). Codistribution was also observed in irregular vacuoles present in the cells transfected with HA-CSQ\textsubscript{1–100} together with CSQ\textsubscript{1–350} (Fig. 2, P and Q). In contrast, when HA-CSQ\textsubscript{1–100} was cotransfected together with CSQ\textsubscript{228–391} (the COOH-terminal 163 amino acids, including the 324–350 sequence), the distribution of both mutants remained diffuse (unpublished data). Taken as a whole, these results confirm that in order to proceed, condensation requires the presence of full-length CSQ\textsubscript{1–391} or CSQ\textsubscript{1–350} mutant molecules expressing at least two binding sites, and that it is inhibited when the CSQ\textsubscript{1–100} mutant, which contains one site only, competes for the binding.

To identify the two binding sites at higher resolution, experiments were set up in which the full-length protein was deleted of short amino acid sequences, or point mutated at strategically located sites. When either site A or site B was deleted, the distribution of the mutants was diffuse (Fig. 3, B and C), coinciding largely with that of CRT (Fig. 3, B\textsubscript{H11032} and C\textsubscript{H11032}). Largely diffuse distribution was also obtained after site-specific mutation of full-length CSQ (Fig. 4 A) at three acidic amino acids in site B, D341A, E344A, and D345A (Fig. 4 C). In contrast, site-specific mutation of three, more proximal glutamic acid residues (E337A, E338A, and E340A) failed to affect the punctate distribution, which remained unchanged with respect to intact CSQ (Fig. 4 B).

**Cellular properties of the CSQ-filled vacuoles**

Additional experiments were aimed at characterizing CSQ puncta as organelles of living cells. In particular, we sought to establish whether CSQ puncta are stable structures or un-
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Figure 5. Fluorescence labeling of living HeLa cells transfected with the GFP/CSQ chimera. (A, A', and A'') Single field of HeLa cells transfected 26 h before with the expression vector encoding the chimera GFP/CSQ. The distribution of the vacuoles rich in CSQ was followed in vivo by the GFP fluorescence at 0 (A), 2 (A'), and 10 min (A''). The square delimits vacuoles that showed some movements during incubation. The same chimera, but with a KDEL signal at the COOH terminus, shows a similar pattern of distribution (B). (C, C', C'', and C''') Single field of cells transfected 26 h before and then treated with the Ca\(^{2+}\) ionophore, ionomycin (1 \(\mu\)M), administered in Ca\(^{2+}\)-free EGTA medium for 0, 20, 60, and 120 min, respectively.

dergo rapid changes in size and/or number, and whether they tend to keep their position or move around within the cell. To this end, L6 and HeLa cells were transiently transfected with the GFP/CSQ chimera. Fig. 5, A–A′′, illustrates the results obtained with HeLa cells. No obvious changes in the average number and size of puncta and of their general pattern of distribution were evident during up to 44 h of incubation. However, at the level of single vacuoles, small and slow displacements were observed especially, but not only, at the periphery of the cell (Fig. 5, A–A′′). Identical results were obtained with the GFP/CSQ chimera containing the COOH-terminal KDEL, i.e., the ER retrieval amino acid sequence (Fig. 5 B). When the cells were treated with nocodazol (33 nM, 1 h) in order to disassemble microtubules, the general distribution and low mobility of puncta were not affected (unpublished data). We conclude that CSQ vacuoles are long-lived, little-moving organelles. Our results exclude that the vacuoles travel rapidly along microtubules, as recently shown for cytoplasmic aggregates of denatured proteins, the so-called aggresomes (Kopito and Sitia, 2000).

CSQ is a Ca\(^{2+}\)-binding and storage protein. Therefore, we investigated whether changes in the ER luminal Ca\(^{2+}\) concentration have any effect on CSQ condensation within intact cells. HeLa or L6 cells, transfected with the GFP/CSQ chimera, were suspended in Ca\(^{2+}\)-free, 1 mM EGTA-containing medium, and exposed to 1 \(\mu\)M ionomycin, a Ca\(^{2+}\) ionophore. Based on the results of our previous studies in the CSQ-L6 cell model, we knew that this treatment induces rapid depletion of the intracellular Ca\(^{2+}\) stores, with transfer of the cation to the extracellular medium (Gatti et al., 1997). However, after up to 2 h incubation with the Ca\(^{2+}\) ionophore, the CSQ immunolabeling appeared unaffected, as shown by the persistence of the typical cytoplasmic puncta (Fig. 5, C–C′′′). We conclude that changes in the ER luminal Ca\(^{2+}\) concentration do not greatly affect the condensed state of CSQ within the vacuoles.

ER retention of CSQ

To investigate whether and to what extent retention of CSQ within the ER and ER-derived vacuoles depends on its condensation competence or a different mechanism(s), batches of L6 and HeLa cells (some stably expressing, others not expressing the full-length CSQ) were transfected with either one of the COOH-terminal–truncated mutants, CSQ\(^{1–350}\) and CSQ\(^{1–324}\), characterized by punctate and diffuse ER distribution, respectively. 46 h after transfection the cells were pulse labeled with \(^{35}\)S)methionine (30 min) and then chased for up to 5 h. Part of the results obtained in HeLa cells can be seen in Fig. 6; similar results from L6 cells are not shown. The labeled, stably transfected full-length CSQ discharged to the medium was only a tiny fraction of that recovered within the cells. Moreover, its rate of discharge during the first hour was <1/50 of that of a typical secretory protein, chromogranin B, transfected to parallel batches of cells and investigated in parallel (unpublished data). The CSQ result was expected because it is consistent with our previous data documenting only minimal transport of the protein to the Golgi complex and the extracellular space (Gatti et al., 1997). The results obtained with the truncated mutant CSQ\(^{1–350}\) documented only a slightly higher release to the medium, which remained very low, irrespective of the coexpression of full-length CSQ. Unexpectedly, the diffusely distributed CSQ\(^{1–324}\) mutant was released at a rate similar to full-length CSQ, i.e., not more, but even less efficiently than CSQ\(^{1–350}\) (Fig. 6). Similar results were obtained by additional experiments carried out in parallel with batches of unlabeled cells, in which release of full-length and truncated CSQ forms was revealed by Western blotting of cells and media, collected at the same time points of the pulse–chase experiments.

Discussion

In this paper, the process of CSQ accumulation within discrete domains of the ER/SR is defined as condensation. This definition was chosen by analogy with the process taking place at the trans-Golgi network, where condensation of secretory proteins gives rise to immature granules (condensing vacuoles). Condensation should not be confused with aggregation, i.e., the state of denatured secretory proteins trapped within the ER lumen because they are able to pass neither the quality control for Golgi transport nor the trans-
location to the cytosol through the ER membrane. These aggregates are composed by proteins extensively cross-linked by disulfide bridges. They have no physiological role but are destined to be disposed of by autophagocytosis (Ellgaard et al., 1999; Kopito and Sitia, 2000).

**CSQ-filled vacuoles as specialized ER domains**

The main task of this work was the identification of the molecular mechanisms responsible for condensation and retention of CSQ within the ER and ER-derived organelles. In striated muscle fibers such a retention was often attributed to the direct binding of the CSQ acidic tail to triadins and junctins, two families of transmembrane proteins that protrude into the cisternal lumen with their long, highly basic COOH-terminal tails (Guo and Campbell, 1995; Jones et al., 1995; Kobayashi et al., 2000; Shin et al., 2000). However, although expression of triadins and junctins is not important for condensation but is instrumental to a subsequent process taking place only in muscle fibers, the docking of the condensed CSQ matrices to the junctional face of SR terminal cisternae.

Another mechanism that has been considered to explain condensation is dependence on the high Ca\(^{2+}\) concentration typical of the ER/SR lumen (He et al., 1993). Indeed, the triadin/junctin interaction with CSQ is released in vitro by Ca\(^{2+}\) withdrawal (Shin et al., 2000). However, in intact L6 or HeLa cells transfected with CSQ, vacuoles remained apparently unchanged after long treatment with the Ca\(^{2+}\) ionophore ionomycin applied in Ca\(^{2+}\)-free medium, a condition known to largely deplete ER stores of their segregated Ca\(^{2+}\) (Gatti et al., 1997). Thus, Ca\(^{2+}\) appears unnecessary for the in vivo maintenance of CSQ in its condensed state.

From the structural point of view, the CSQ-containing vacuoles appeared heterogeneous. In fact, some were regularly spherical, dense, and compact, whereas others (especially those containing the truncated CSQ1–350 mutant) were irregular, with a content alternating dense and clear areas.

Content heterogeneity is not unique to CSQ-containing vacuoles, but has been observed in the content of various hormone secretion granules (see for example Orci, 1982), possibly due to peculiar aspects of their condensation processes. The ultimate significance of content heterogeneity remains undefined.

Our previous studies (Gatti et al., 1997) had excluded the CSQ-containing vacuoles to be lysosomes, endosomes, and part of the Golgi complex. In contrast, vacuoles were identified as discrete, specialized domains of the ER, similar in a few aspects to the SR terminal cisternae and resembling more closely the CSQ-rich vacuoles that appear within myocytes of transgenic mice overexpressing the protein (Jones et al., 1998; Sato et al., 1998). An additional property of the CSQ vacuoles revealed in the present work, low mobility throughout the cytoplasm, was not due to their interaction with microtubules, because it was unaffected by depolymerization induced by nocodazol. An alternative possibility is the direct interaction with the ER network, a system in continuous protein exchange with the vacuoles (Gatti et al., 1997) that as a whole undergoes only slow oscillations and retractions within the cells (Terasaki and Jaffe, 1993).

**The CSQ condensation process**

The molecular mechanisms of CSQ condensation were investigated in a large spectrum of mutants expressed in both L6 and HeLa cells. The first series of experiments was carried out by using chimeras of CSQ with another ER luminal Ca\(^{2+}\)-binding protein, CRT. In spite of their low degree of homology, luminal distribution, and function (CRT is a chaperone diffuse throughout the whole ER) (Michalak et al., 1999; Molinari and Helenius, 2000), CSQ and CRT share similarities in structure and low-affinity Ca\(^{2+}\)-binding properties. These similarities are particularly evident at their highly acidic COOH-terminal tail (70–80 amino acids), a region considered of importance also for the non-KDEL mechanism of retention in the ER (Sonichsen et al., 1994). However, in both cell types investigated the results clearly excluded CSQ condensation to depend on the acidic tail alone. In fact, both the CRT/CSQ and the CSQ/CRT chimeras remained diffusely distributed throughout the ER lumen. These data strongly suggest the existence of not one but at least two condensation sites, one located in the COOH-terminal tail, the other elsewhere in the CSQ molecule. The existence of an NH\(_2\)-terminal site was confirmed by coexpression results with the GFP/CSQ chimera and the COOH-terminal–truncated mutant.
CSQ^1–100. In fact, depending on their expression ratio, the two forms were seen colocalized within either the ER cisternae or the vacuoles, strongly suggesting their direct binding. Finally, the existence of two binding sites was confirmed by the results with truncated and point-mutated constructs that lead to the identification of site A, corresponding to the short NH_2-terminal 15–amino acid sequence, and site B, located COOH-terminal of residue 337, with critical involvement of the CSQ acidic residues 341, 344, and 345.

The CSQ condensation model

Based on the specific information reported so far, on previous biochemical results (Cala and Jones, 1983; Maguire et al., 1997; Zhang et al., 1997) and the known crystal structure (Wang et al., 1998), we have developed a model for CSQ condensation using the Biopolymer option in InsightII software (Fig. 7). The x-ray results had revealed that the protein may form “front-to-front” and “back-to-back” dimers (Fig. 7, F-F and B-B, respectively; Wang et al., 1998) in which the NH_2 terminus and the COOH terminus could either fit into a groove between domains 1 and 3 or face away from the dimer interface, thereby enabling them to interact with other CSQ molecules. From these data, models of possible higher oligomers were developed. Only two regularly repeating structures showing the expected NH_2- and COOH-terminal alignment were identified. The first is the front-to front and back-to-back oligomer revealed also by x-ray (Wang et al., 1998), the other is a “front-to-back” structure (Fig. 7, potential) that emerged from the analysis of the present data. The localization near the COOH terminus of the α-helical region including the critical residues, D341, D344, and D345, far away from the NH_2-terminal sequence, appears compatible with its direct involvement in the CSQ–CSQ intermolecular binding. The polymers established according to the above models most likely correspond to the CSQ filaments revealed by electron microscopy within deep-etched SR terminal cisternae (Franzini-Armstrong et al., 1987).

CSQ retention

Our pulse–chase release experiments confirmed that during cell life, only a minimal fraction of the full-length CSQ^1–391 is released to the medium, whereas the rest is retained within the cell (Gatti et al., 1997). If retention were a consequence of condensation, it should be shown only by full-length CSQ^1–391 and the COOH-terminal–truncated mutant CSQ^1–350, and does not extend to the diffusely distributed mutant CSQ^1–324 that lacks one of the two condensation sites. In contrast, our results also demonstrate that the diffuse CSQ^1–324 mutant is released at rates at least 50-fold slower than those of a typical secretory protein, chromogranin B, that in the cells investigated is known to travel along the pathway most likely followed by the released fraction of CSQ, i.e., constitutive secretion. Therefore, we conclude that retention within the ER is a property that CSQ possesses independently of its condensation competence. In view of the similar results obtained with the full-length and the COOH-terminal–truncated constructs, the site(s) that prevents CSQ transport out of the ER appears to reside not in the COOH-terminal acidic tail but in the globular domains of the protein. The role of the retention site(s) might be important, especially for the soluble, diffusely distributed ER/SR pool in equilibrium with the condensed matrices of vacuoles and muscle SR terminal cisternae.

In conclusion, intraluminal retention, condensation, and specific distribution of CSQ are shown here to be sustained by multiple and independent mechanisms. The first mechanism is a molecular retention step, apparently similar to the KDEL-independent process of other luminal proteins (Sonichsen et al., 1994; Monnat et al., 2000). The second is condensation, which depends on specific, molecularly identified A and B sites. The third (in striated muscle fibers but not in the other cells where CSQ is expressed) is the specific docking of condensed matrices, most likely by the membrane proteins associated to ryanodine receptors, i.e., triadins and junctions (Guo and Campbell, 1995; Jones et al., 1995; Zhang et al., 2001).

Condensation is not an exclusive property of CSQ, but appears to also take place with other ER proteins, single or as mixtures, in both animal and vegetal cells (see, for example, Titorenko and Rachubinski, 1998; Choi et al., 2000; Chriseps and Herman, 2000). Moreover, condensation does occur in other intracellular compartments, for example at the trans-Golgi network of secretory cells (Chanat and
Materials and methods

Cell lines and antibodies

The L6 myogenic cell line (Yaffe, 1973) and HeLa cells were from the American Type Culture Collection. Rabbit anti–chicken CRT (Perrin et al., 1991) was gifts of Drs. D.O. Clegg (Neuroscience Research Institute, University of California, Santa Barbara, CA) and H.-D. Soling (Max-Planck Institute for Biophysical Chemistry, Göttingen, Germany), respectively. The mAb against the HA tag (mAb 12CA5) was from Santa Cruz Biotechnology, murine IgGs were from Jackson ImmunoResearch Laboratories, and CY2- and rhodamine-labeled goat IgGs were against rabbit and mouse IgGs from Jackson Immunoresearch Laboratories.

PCR amplification

Degeneracy of the NH2-terminal part of CSQ was amplified using the following primers: 5'-ATCGGCTAGCATGCTGCTCC-3' and 5'-ATCGGGTACCCCTTTCCAGAAAGCACATCCT-3' and the 3'-oligooxyxynucleotide was 5'-CGATCCAGTCCGCCAGCCGACTGTGGCCAGC-3'. For specific mutation (site B) of E337A, E338A, and E340A, the 5'-ATCGGCTAGCATGCTGCTCC-3' and the 3'-oligooxyxynucleotide was 5'-CGATCCAGTCCGCCAGCCGACTGTGGCCAGC-3'. For site-specific mutation (site B) of D341A, E344A, E345A, the 5'-ATCGGCTAGCATGCTGCTCC-3' and the 3'-oligooxyxynucleotide 5'-GGAGCTGGAGGCCTG-GATCCAGTCCGCCAGCCGACTGTGGCCAGC-3'. For site-specific mutation (site B) of KDEL signal using as 3'-oligooxyxynucleotide 5'-TAGAAATCTCATCTACATCATCATCAT-3'.

Generation of GFP/CQ proteins

The plasmid pB6ST-C containing cDNA encoding GFP was purchased from CLONTECH Laboratories, Inc. To create expression plasmids encoding GFP/CQ chimera, the signal sequence of CQ was first inserted at the 5'-end of GFP cDNA; this was followed by cloning CQ cDNA at the 3'-end of GFP cDNA. CSQ cDNA was generated by PCR-driven amplification using as 5'-oligooxyxynucleotide 5'-AAATTCGAGAAGGGGGCTATTCAACG-TACATC-3' and the 3'-oligooxyxynucleotide 5'-TTAAGCGGCCGCTCATGCTACATCATCACATCGGCTAGCATGCTGCTCC-3'. For site-specific mutation (site B) of D341A, E344A, E345A, the 5'-ATCGGCTAGCATGCTGCTCC-3' and the 3'-oligooxyxynucleotide 5'-GGAGCTGGAGGCCTG-GATCCAGTCCGCCAGCCGACTGTGGCCAGC-3'.

Cell culture, transient transfections, immunofluorescence, electron microscopy, and immunoelectron microscopy

L6 myoblast and HeLa cells were transfected as described previously (Gatti et al., 1997). Expression vectors for HA-tagged CRT and CSQ were described previously (Bastianutto et al., 1995; Nori et al., 1997). SDS-PAGE, Western blot analysis, immunofluorescence, electron microscopy of Epon-embedded sections, immunogold labeling of ultrathin cryosections, and LR White–embedded samples were carried out as described in our previous studies (Gatti et al., 1997).

Release of CSQ and CSQ mutants to the incubation medium

Multiple (two per time point) monolayers of L6 and HeLa cells stably transfected with full-length CSQ (Gatti et al., 1997) were transiently transfected with either CSQQ1–391 or CSQ3–1391. 46 h after transfection the monolayers were washed twice and covered with fresh DMEM without serum and methionine, and supplemented with 140 μCi/ml of [35S]methionine. After 30 min labeling at 37°C (pulse), monolayers were washed again and incubations were continued in a nonradioactive DMEM with serum (chase) for 0, 60, 120, 180, and 300 min. Media and detached cells were collected separately at the time points indicated above, and the various CSQ forms were immunoprecipitated with anti-CSQ antibody (Ab). The immunoprecipitates were run on SDS-PAGE gels. Additional experiments were carried out with cell preparations stably transfected with either full-length CSQ or CSQ3–1391. After staining (Coomasie blue) and soaking in the amplifier, the gels were autoradiographed and the radioactivity of CSQ bands was assayed by microdensitometry. Additional batches of cells, transfected with the same CSQ forms as above, were incubated according to the same protocol, although without [35S]methionine. At each time point, cells and media were collected and the release of full-length CSQ and mutants was established by Western blotting.

In vivo analysis of CSQ vacuoles

Fluorescent vacuoles were generated by transient transfection of GFP/CQ with or without KDEL constructs. 26–44 h after transfection, washed monolayers were covered with Krebs-Ringer medium (no serum) and transferred to the fluorescence microscope (10×). Analyses for GFP distribution were carried out for the times indicated in the legend to Fig. 5. In parallel experiments, monolayers were covered with Krebs-Ringer medium without Ca2⁺ and containing 1 mM EGTA and 1 μM of the Ca2⁺ ionophore, ionomycin, were examined in the fluorescent microscope up to 120 min.

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