Calsequestrin targeting to sarcoplasmic reticulum of skeletal muscle fibers

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Nori, Alessandra, Giorgia Valle, Elena Bortoloso, Federica Turcato, and Pompeo Volpe. Calsequestrin targeting to sarcoplasmic reticulum of skeletal muscle fibers. Am J Physiol Cell Physiol 291: C245–C253, 2006. First published March 29, 2006; doi:10.1152/ajpcell.00370.2005.—Calsequestrin (CS) is the low-affinity, high-capacity calcium binding protein segregated to the lumen of terminal cisternae (TC) of the sarcoplasmic reticulum (SR). The physiological role of CS in controlling calcium release from the SR depends on both its intrinsic properties and its localization. The mechanisms of CS targeting were investigated in skeletal muscle fibers and C2C12 myotubes, a model of SR differentiation, with four deletion mutants of epitope (hemagglutinin, HA)-tagged CS: CS-HAΔ24, CS-HAΔ2D, CS-HAΔ3D, and CS-HAΔHT, a double mutant of the NH2 terminus and domain III. As judged by immunofluorescence of transfected skeletal muscle fibers, only the double CS-HA mutant showed a homogeneous distribution at the sarcomeric I band, i.e., it did not segregate to TC. As shown by subfractionation of microsomes derived from transfected skeletal muscles, CS-HAΔHT was largely associated to longitudinal SR whereas CS-HAΔ2D and CS-HAΔ2D were not sorted to developing SR. Condensation competence, a property referable to CS oligomerization, was monitored for the several CS-HA mutants in C2C12 myoblasts, and only CS-HAΔ3D was found able to condense. Together, the results indicate that 1) there are at least two targeting sequences at the NH2 terminus and domain III of CS, 2) SR-specific target and structural information is contained in these sequences, 3) heterologous interactions with junctional SR proteins are relevant for segregation, 4) homologous CS-CS interactions are involved in the overall targeting process, and 5) different targeting mechanisms prevail depending on the stage of SR differentiation.

protein-protein interactions; oligomerization; intracellular sorting

THE SARCOPLASMIC RETICULUM (SR), a subcompartment of the endoplasmic reticulum (ER), is molecularly specialized for the control of cytosolic Ca2+ concentration [Ca2+]i, and of the contraction-relaxation cycle in skeletal muscle fibers (9). SR proteins are mainly devoted to Ca2+ uptake [sarco(endo)plasmic reticulum Ca2+-ATPase, SERCA], intralumen low-affinity, high-capacity Ca2+ storage (calsequestrin, CS), and Ca2+ release to the myoplasm (Ca2+ release channel or Ryanodine receptor, RyR). SR proteins are developmentally regulated and topologically segregated to either terminal cisternae (TC) of SR, e.g., RyR, CS, triadin (TD), and junctin (JC), or longitudinal SR (LSR), e.g., SERCA (1, 8).

Mechanisms for intracellular targeting of SR proteins in skeletal muscle fibers are largely unknown; with respect to CS, such mechanisms are referable to retention and sorting in ER, routing through different cell compartments, and segregation to TC, depending on CS concentration and docking. CS is specifically targeted to the junctional face of TC (9, 34), also known as junctional SR (jSR). In recent years, classic as well as muscle-specific intracellular pathways for protein targeting have been described and characterized not only in adult skeletal muscle fibers (14, 15, 32, 33) but also during myogenesis (31).

Polymerization, an intrinsic property of CS, is implicated in Ca2+ binding (30, 39) and CS condensation (11). TD and JC have been identified by in vitro experiments (12, 35, 41) as potential CS anchor proteins; recently, a specific KEKE motif on cardiac TD has been reported to be essential for in vitro binding to CS via polar zippers (16). Although several hypotheses have been put forward to account for CS segregation (pure electrostatic interactions, heterologous and/or homologous protein-protein interactions) and different CS domains, spanning almost the entire molecule, have been implied, no data exist on the CS sequences responsible for segregation in vivo. Other, yet unresolved questions concern the mechanisms by which CS reaches TC, i.e., CS routing, via either coated vesicles (23, 36) or intraluminal diffusion mediated by putative ER/SR continuities (38), and is docked to jSR. For instance, Gatti et al. (10) reported that CS, overexpressed in L6 myoblasts, is excluded altogether from routing through the Golgi complex.

Otte and Barlowe (29) recently identified a specific motif driving receptor-mediated export from ER of soluble proteins in coat protein complex II (COPII)-derived vesicles. The presence of the I-L-V motif in CS and its conservation among different animal species support our previous in vivo findings that COPII-mediated export is involved in CS targeting to TC (23). The physiological relevance of such a pathway is indirectly highlighted by the expression of the specific receptor (Surf-4) in mammalian skeletal muscles as well as in the body wall muscle of Caenorhabditis elegans (13); moreover, functional knockdown of Surf-4 induces larval arrest, thus suggesting that the Surf-4 gene may have an essential role in development. In skeletal muscle fibers, CS routing may enter this pathway because synthesis of CS appears to be restricted to perinuclear ER and underneath the plasma membrane, as judged by the compartmentalized distribution of CS mRNA (22), i.e., massive export from ER and subsequent transport to TC appear necessary. However, a homogeneous distribution of CS mRNA has been found in myotubes (22), implying that different mechanisms could be involved in CS targeting in either mature, fully differentiated skeletal muscle fibers, i.e., under steady-state conditions, or during biogenesis and SR differentiation, i.e., under non-steady-state conditions.

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To identify the specific sequences of CS responsible for targeting in vivo and the molecular mechanisms at work, four deletion mutants of CS-hemagglutinin (HA) were engineered and expressed in three different cellular systems in which the SR displays different levels of molecular differentiation and morphological organization: 1) C2C12 myoblasts, myogenic mononucleated cell precursors of myotubes; 2) C412 myotubes, an established experimental system suitable for studying SR during muscle differentiation (1, 18); and 3) in vivo transfected skeletal muscle fibers of the adult rat, containing fully differentiated and morphologically recognizable TC to which endogenous CS is targeted (25–27). By comparing the distribution of CS-HA mutants with that of endogenous CS, identification of two critical target sequences of CS was accomplished; evidence was also accrued as to different targeting mechanisms of CS, either during SR differentiation or at steady state, i.e., in differentiated skeletal muscle fibers.

**MATERIALS AND METHODS

Construction of cDNAs Coding for CS-HA Mutants**

The cDNA corresponding to CS-HA and pBSK⁺/CSHA were developed as previously described (25, 27). Schematic representations of CS-HA mutants are depicted in Fig. 1. CS-HAΔ3D [deletion of the cDNA coding for amino acids (aa) 230–367 corresponding to domain III] was produced by restriction of pBSK⁺/CSHA with AccI and subsequent addition of the HA tag by ligation of the adaptor duplex obtained by annealing the primer 5'-CTAATCAGGAACATCATAGGT-3' with the primer 5'-CTCTAGGTCCT-CTCTAGGT-3'. The resulting plasmid was digested with NotI-XhoI and the cDNA inserted in the eukaryotic expression vector pCDNA3 in the same sites. For CS-HAΔ2D (deletion of the cDNA coding for aa 85–263 including domain II), the EcoRI linker 5'-CGGAATTCG-3' was inserted with blunt-end ligation in the BamHI site of pBSK⁺/CS-HA. Digestion with EcoRI and consecutive religation of the plasmid allowed the elimination of the 537-bp EcoRI-EcoRI fragment that encodes for domain II. For CS-HAΔ24NH₂ (deletion of the first 24 aa at the NH₂ terminus) a synthetic cDNA corresponding to the CS signal sequence was obtained by PCR from the vector pBSK⁺/CS-HA. The resulting fragment was inserted in the EcoRI site of pBSK⁺ and subsequently excised by AflIII and ligated to the AflIII-cut pBSK⁺/CS-HA. The mutated cDNA was then inserted in the NotI-XhoI sites of pCDNA3. For CS-HAΔHT (a double mutant lacking both domain III and the first 24 aa at the NH₂ terminus) the strategy adopted for CS-HAΔ24NH₂ was applied to the mutant CS-HAΔ3D in the intermediate vector pBSK⁺.

In Vivo Transfection of Skeletal Muscle Fibers

Male adult Wistar rats (~250-g body wt) were anesthetized with ketamine (1.5 mg/100 g body wt). The right soleus muscles were exposed and injected with 0.06 ml of a saline solution containing 50 μg of either plasmid cDNA. Electroporation was carried out with a BTX ECM 830 square-wave pulse generator and the protocol described by Nori et al. (26), i.e., pulse stimulation by field electrodes (220 V/linear cm) with six 20-ms pulses at 200-ms intervals. Treated rats were killed 14 days after surgery.

**Cell Cultures**

C2C12 myoblasts (40) were grown in DMEM supplemented with 10% fetal calf serum (proliferation medium). Cells were seeded into petri dishes or individual wells of a 24-well plate containing 13-mm glass, 0.02% gelatin-coated coverslips. When approaching confluence, C2C12 myoblasts were induced to differentiate and fuse into multinucleated myotubes by changing the proliferation medium to DMEM with 2% horse serum (differentiation medium).

**Generation of Transient Transfectants of C2C12 Myoblasts and C2C12 Myotubes**

The cells were transfected during the growing phase on gelatin-coated coverslips or petri dishes; when they attained either ~40% or ~60% confluence, for obtaining myoblasts or myotubes, respectively, a transfection mixture containing three parts Fugene (Roche) and one part plasmid in DMEM was added to the growth medium. Fixation of myoblasts was carried out 24 h later, whereas the transfection mixture was replaced with differentiation medium 16–18 h later and myotubes were fixed 4 days after transfection.

**Immunofluorescence**

For soleus muscles, 6-μm longitudinal sections were obtained and incubated with primary antibodies at room temperature for 60 min as described previously (27). After extensive washing, muscle sections were incubated for 30 min with either Cy2-conjugated anti-mouse (Chemicon) or rhodamine isothiocyanate anti-rabbit (DAKO) antibodies. Myoblasts and myotubes were fixed in 4% paraformaldehyde, 240 mM phosphate buffer, pH 7.4, for 30 min, and permeabilized with 0.3% Triton X-100, 20 mM phosphate buffer, pH 7.4, 0.45 M NaCl, and 15% goat serum (incubation buffer) for 30 min. Incubation with primary antibodies was performed at room temperature for 60 min in incubation buffer. After 60-min washing, cells were incubated for 30 min with rhodamine isothiocyanate-, Cy2 (Chemicon), or fluorescein-conjugated anti-mouse (Sigma) or rhodamine anti-rabbit (DAKO) antibodies. Immunofluorescence sections were analyzed under a Leica HC microscope (23).

Fluorescence intensity profiles were analyzed by computerized imaging software (MicroImage, Casti Imaging, Venice, Italy). The fluorescence intensity signal was obtained on the merged images, for each fluorophore (peaks) was defined as the period. The distance between the two points having maximum fluorescence intensity for each fluorophore (peaks) was defined as the period.

**Subcellular Fractionation**

Membrane fractions were prepared from control (not transfected) soleus muscles and from muscles transfected with either CS-HA or CS-HAΔHT according to Saito et al. (34) with slight modifications. To obtain a large amount of microsomes, homogenates underwent three consecutive homogenization cycles and ensuing supernatants were combined before being spun down at 100,000 g. Subfractionation was carried out by isopycnic sucrose gradient centrifugation as described by Saito et al. (34). Protein concentration was determined according to Lowry et al. (19).

**Gel Electrophoresis and Western Blot Analysis**

SDS-PAGE was performed on 5%-10% linear gradient gel according to Laemmli (17). Depending on the subsequent biochemical
analysis, the amount of protein per lane was 50 μg for Stains All staining, 100 μg for Western blot with anti-RyR1 antibodies, 8 μg for immunoblot with anti-SERCA2 and anti-CS antibodies, and 10 and 50 μg for immunoblot with anti-HA antibodies in muscles transfected with either CS-HA or CS-HADHT, respectively. After electrophoretic separation, proteins were either stained with Stains All staining or transferred from the gel onto nitrocellulose membranes. Densitometric scanning was performed on a Bio-Rad Molecular Imager (model GS-250) with Molecular Analyst software (Bio-Rad).

Primary Antibodies

Mouse anti-CS monoclonal antibodies were from Affinity BioReagents, rabbit anti-HA polyclonal antibodies from Santa Cruz Biotechnology, sheep anti-RyR1 polyclonal antibodies from Upstate Biotechnology, and rabbit anti-SERCA2a polyclonal antibodies from Badrilla; anti-rabbit CS polyclonal antibodies were raised in chickens.

RESULTS

A combined molecular and cell biology approach was implemented to unravel the CS sequences responsible for targeting in vivo. Four deletion mutants of the epitope HA-tagged skeletal muscle CS (Fig. 1) were thus developed to interfere with homologous and/or heterologous interactions of CS. The rationale for deletion was based on knowledge that 1) domain II contains some sequences capable of front-to-front dimer formation (39), 2) domain III contains two disordered sequences putatively involved in heterologous interactions (7) and additional sequences capable of back-to-back dimer formation (39), 3) the first 24 aa at the NH2 terminus are reported to be involved in heterologous interactions in vitro (16), and 4) the fourth mutant combines deletion of the NH2 terminus and domain III.

The effects of such deletions were investigated in three different experimental systems: 1) rat soleus muscle, a model displaying fully differentiated and morphologically recognizable TC to which endogenous CS is targeted (27); 2) C2C12 myotubes, a model of differentiating SR; and 3) C2C12 myoblasts, myogenic mononucleated cell precursors of multinucleated myotubes. Moreover, subcellular localization studies were complemented with biochemical studies of membrane subfractions to identify the specific SR compartment containing CS, CS-HA, or CS-HA mutants.

Combined Deletion of NH2 Terminus and Domain III Disrupts Targeting of CS-HA to TC in Skeletal Muscle Fibers

Expression of CS-HA mutants was obtained by electroporation of the corresponding cDNAs in rat soleus muscles. The subcellular distribution of CS-HA and of deletion mutants was compared with that of endogenous CS by double immunofluorescence of soleus muscle longitudinal sections 14 days after transfection; the results are summarized in Fig. 2. Localization of exogenous proteins was monitored with anti-epitope (HA)
SERCA2 was enriched in muscle CS was metachromatically stained in blue with Stains-All, and SERCA2 for LSR. In Fig. 3B, transfected) slow-twitch muscles. Three well-known markers by isopycnic sucrose gradient centrifugation from control (non-transfected) were partially separated in skeletal muscle fibers (27). Overlap with endogenous CS was also observed for those CS-HA mutants bearing deletions of domain III (Fig. 2D), NH2 terminus (Fig. 2G), and domain II (Fig. 2O). In contrast, CS-HAΔHT, LSR whereas CS-HA, was not enriched in discrete structures referable to TC and appeared to be retained in an adjacent compartment encompassing the sarcomeric I band (see Fig. 2N, inset). Whether or not CS-HA and CS-HAΔHT were retained in membrane compartments with distinct biophysical properties was directly investigated after sucrose gradient purification of microsomes obtained from rat soleus muscles previously transfected with cDNAs encoding either CS-HA or CS-specific markers. Because subfractions R3 and R4 were relatively enriched in both TC markers, CS and RyR1, we infer that in soleus muscle R3 and R4 are referable to heavy/cisternal SR (HSR) whereas R2 mainly represents light/longitudinal SR (LSR; cf. Ref. 20).

As judged by immunofluorescence, CS and HA labeling were partially separated in skeletal muscle fibers transiently transfected with CS-HAΔHT (cf. Fig. 2, L–N); CS-HAΔHT, at variance with CS-HA, was not enriched in discrete structures referable to TC and appeared to be retained in an adjacent compartment encompassing the sarcomeric I band (see Fig. 2N, inset). Whether or not CS-HA and CS-HAΔHT were retained in membrane compartments with distinct biophysical properties was directly investigated after sucrose gradient purification of microsomes obtained from rat soleus muscles previously transfected with cDNAs encoding either CS-HA or CS-

Subcellular Fractionation of Transfected Soleus Muscles: CS-HAΔHT Dissociates from Endogenous CS and Is Mainly Associated to LSR Markers

Saito et al. (34) developed a method by which highly purified membrane fractions, referable to TC, LSR, or T tubule/plasma membrane, are obtained from rabbit fast-twitch muscles. A similar purification procedure was applied to slow-twitch muscles of the rat, as a first step toward characterization of purified membrane fractions obtained from transfected soleus muscles.

Figure 3 shows the representative electrophoretic pattern and the distribution of specific SR markers (Fig. 3, A and B, respectively) of four membrane subfractions (R1–R4) obtained by isopycnic sucrose gradient centrifugation from control (non-transfected) slow-twitch muscles. Three well-known markers were chosen to characterize the subfractions: CS and RyR1 for TC and SERCA2 for LSR. In Fig. 3A, endogenous skeletal muscle CS was metachromatically stained in blue with Stains-All and was found to be enriched in subfractions R3 and R4; RyR1 was likewise enriched in both subfractions R3 and R4, as judged by Western blot (Fig. 3B, top). On the other hand, SERCA2 was enriched in subfraction R2, as judged by Western blot (Fig. 3B, bottom). These data show that in slow-twitch muscles vesicles belonging to distinct SR compartments display different densities and can be identified by means of
HAΔHT. The distribution of HSR vs. LSR endogenous markers among the four subfractions was not significantly affected by expression of either CS-HAΔHT or CS-HA, as shown by Western blot with anti-SERCA2 and anti-CS antibodies (Fig. 4A, top). The distribution of the HA label among the four subfractions was, instead, quite different, being more represented in subfraction R2 obtained from CS-HAΔHT-transfected muscle (Fig. 4A, bottom).

A quantitative densitometric analysis was performed and was calculated as the ratio between the HA label and the LSR compartment-specific marker, i.e., endogenous SERCA2, which is known not to interact with CS either in vivo or in vitro. Figure 4B shows average histograms of the Western blots obtained from four subfractionation experiments. The mean values of each LSR fraction show that the CS-HAΔHT-to-SERCA2 ratio was significantly higher compared with the CS-HA-to-SERCA2 ratio. These results demonstrate that CS-HAΔHT had a significantly different fractionation pattern compared with either exogenous CS-HA or endogenous CS; i.e., CS-HAΔHT was preferentially contained in LSR vesicles enriched in SERCA2.

Thus biochemical data agreed well with immunofluorescence data, and both suggest that the intracellular compartment proximal but distinct from TC, possibly LSR, was the main site of CS-HAΔHT concentration. Retention of CS-HAΔHT to ER cannot be ruled out with certainty, because Gatti et al. (10, 11) suggested that an ER retention signal, possibly spared in CS-HAΔHT, was located in the globular domains of CS.

**NH2 Terminus and Domain II of CS-HA Confer Condensation Competence**

Gatti et al. (11) identified condensation as a specific property of cardiac CS and suggested that condensation, i.e., homologous CS-CS interactions, was a key mechanism in promoting specialization of the ER lumen and, accordingly, of SR compartments. Condensation competence of the four CS-HA mutants was tested in C2C12 myoblasts, i.e., in the absence of endogenous CS as well as known CS anchors, to ascertain whether the NH2 terminus or domain III is involved in condensation of skeletal muscle CS.

Figure 5 shows that condensation ability is displayed only by one of the CS-HA mutants and by the recombinant CS-HA. Figure 5B shows CS-HAΔ3D large clusters, comparable to those observed for cardiac CS (11) and recombinant skeletal CS-HA (Fig. 5A); in contrast, reticular, ER-like, diffuse distribution was observed for CS-HAΔ24NH2, CS-HAΔHT, and CS-HAΔ2D (Fig. 5, C–E). Thus CS-HAΔ24NH2 and CS-HAΔHT were unable to condense in discrete structures in the absence of endogenous CS and CS anchors. By comparing CS-HAΔ24NH2 and CS-HAΔHT with CS-HAΔ3D, it emerges that the condensation competence of skeletal muscle CS within the ER lumen depends on two sequences, one spanning 24 aa...
at the NH₂ terminus and the other endowed in domain II, because their individual deletion was sufficient to confer a diffuse distribution pattern to the corresponding mutant.

**NH₂ Terminus of CS-HA Is Not Essential for Targeting to Developing SR in C₂C₁₂ Myotubes**

Because deletion of the NH₂ terminus abolished condensation and did not affect per se targeting to TC in skeletal muscle fibers, the subcellular localization of CS-HAΔ24NH₂, CS-HAΔHT, CS-HA3D, and CS-HAΔ2D was compared with that of endogenous CS in a third experimental system, C₂C₁₂ myotubes, that constitutes a well-known experimental system suitable for studying SR during muscle differentiation (1, 18) and adequate to assess whether condensation is involved in early specialization of SR.

Figure 6 shows that HA (Fig. 6A) and CS (Fig. 6B) colocalized on transfection with CS-HA. On the other hand, CS-HAΔHT (Fig. 6E), CS-HAΔ2D (Fig. 6G), and CS-HA3D (not shown) displayed a homogeneous, ER-like distribution and did not colocalize with either endogenous CS (compare Fig. 6E with F and G with H, respectively) or TD (results not shown). Moreover, distribution of CS-HAΔ24NH₂ was quite different (Fig. 6C): HA labeling was partly organized in clusters (arrows), also positive for both endogenous CS (Fig. 6D) and TD (not shown), and partly was diffuse.

Thus a large deletion including domain II affected both condensation and targeting to developing SR; deletion of domain III did not affect condensation but disrupted targeting to developing SR, whereas deletion of the NH₂ terminus abolished condensation and modified intracellular CS distribution without precluding CS targeting to discrete regions of developing SR. Together, these data indicate that 1) at least two sequences were involved in CS targeting to developing SR, that located in domain III being dominant over the NH₂ terminus sequence, as far as segregation is concerned; 2) targeting required additional structural information endowed within domain II and the NH₂ terminus; and 3) condensation was involved in the overall CS targeting process.

The results obtained with the three different model systems—muscle fibers, myoblasts and myotubes—are summarized in Table 1.

**DISCUSSION**

Two domains of CS relevant for its targeting to TC are identified for the first time in vivo: in skeletal muscle fibers, CS-HAΔHT, the mutant bearing a double deletion of the entire domain III and of the first 24 aa at the NH₂ terminus, did not segregate to TC, whereas CS-HA3D and CS-HAΔ24NH₂, each bearing a single deletion, were correctly segregated to TC. The mechanistic role of both domains is supported by experiments carried out in two additional model systems. In C₂C₁₂ myotubes, CS-HAΔHT was not sorted along with endogenous CS to developing SR; in C₂C₁₂ myoblasts, the absence of both domain III and the first 24 aa at the NH₂ terminus inhibited CS condensation, i.e., homologous CS-CS interactions.

Previous work from our laboratory has shown that 1) CS targeting to TC of skeletal muscle fibers is not affected by either elimination of glycosylation and phosphorylation sites (24, 28) or short deletions at the COOH terminus (25) and 2) CS targeting depends on active export from ER (23). Within this context, two issues will be discussed: Can the NH₂ terminus and domain III of CS be defined as sensu stricto target sequences to TC of skeletal muscle fibers and developing SR of C₂C₁₂ myotubes? Which are the targeting mechanisms in vivo?
In vitro studies have shown that the NH2 terminus and domain III of CS contain target sequences to TC of skeletal muscle fibers; the targeting properties of the NH2 terminus and domain III appear to be typical of organelle-specific target sequences, whereas structural information is included at the NH2 terminus and in domain II. Thus targeting of all possible CS oligomers that expose alternatively domain III concern the specific docking to jSR. It is also entirely possible that CS interacts with different anchor proteins during SR differentiation, e.g., transition of TD isoforms to ER-like distribution and ER-like distribution, respectively (see Table 1). In skeletal muscle fibers, instead, the NH2 terminus exerted a targeting role only in the absence of domain III and domain II did not disrupt targeting. The straightforward interpretation of these results is that two noncontinuous CS domains allow correct localization of CS in TC of skeletal muscle fibers; the targeting properties of the NH2 terminus and domain III appear to be typical of organelle-specific target sequences, and targeting seems to be driven by either of two signals and unrelated to CS tertiary structure.

In C2C12 myotubes, double deletion of the NH2 terminus and domain III disrupted CS clustering in SR, i.e., a result fully consistent with that obtained in muscle fibers. On the other hand, individual deletion of domain III and domain II disrupted targeting to developing SR, in contrast with the observations made in muscle fibers, whereas single deletion of the NH2 terminus led to an ER-like distribution of CS-HAΔ24NH2 but allowed targeting to developing SR. A plausible interpretation of the data on C2C12 myotubes is that domain III contains organelle-specific target sequences, whereas structural information is included at the NH2 terminus and in domain II. Thus multiple targeting mechanisms might handle such sequences in vivo depending on the state of SR differentiation, e.g., muscle fibers vs. C2C12 myotubes, as discussed below.

**Molecular Mechanisms Involved in CS Localization in Vivo**

Two targeting mechanisms for CS can be postulated in vivo and deserve to be discussed: heterologous interactions (CS with CS anchors) and homologous interactions (oligomerization-mediated condensation).

**Heterologous interactions (receptor-mediated protein targeting).** In vitro studies have shown that the NH2 terminus and the COOH terminus are individually qualified for heterologous interactions. A potential site on cardiac CS has been proposed to be the highly charged NH2 terminus able to establish a polar zipper interaction with KEKE sequences on TD and JC (16, 30, 39, 41). For skeletal muscle CS, multiple sites have been proposed as putative binding sequences for either JC or TD: Shin et al. (35) identified a short asp-rich region at the COOH terminus, Collins et al. (6) the region spanning aa 96–191, and Dunker et al. (7) a disordered sequence corresponding to aa 327–333.

The present results and previous work (25) demonstrate that, during development, the asp-rich region and aa 96–191 were not sufficient for correct targeting and an alternative and/or complementary sequence encompassing aa 230–354 should be involved. Moreover, deletion of the NH2 terminus did not affect targeting per se. The plausible interpretation is that, during development, a dominant signal encompassing aa 230–354 controls targeting via heterologous interactions. The NH2 terminus and domain II appear to be involved in other mechanisms.

On the other hand, in skeletal muscle fibers both domain III and the NH2 terminus behaved as redundant target sequences: because both sequences are always copresent and CS is known to undergo multiple Ca2+-induced conformational changes (from monomers to dimers and polymers), the physiological implication of our findings is that redundancy allows CS targeting of all possible CS oligomers that expose alternatively one or the other target sequence. Heterologous interactions have been proposed as a relevant mechanism for both CS docking and routing. Because an ER exiting sequence mediated by COPII (29) is present in CS, it is conceivable that both the NH2 terminus and the COOH terminus sequences within domain III concern the specific docking to jSR. It is also entirely possible that CS interacts with different anchor proteins during SR differentiation, e.g., transition of TD isoforms Trisk 95 and Trisk 51 (37) might occur, so that the relative role of CS targeting domains might be correspondingly modified.

**CS oligomerization.** We demonstrate that the NH2 terminus and domain II of CS were not only causally related to CS condensation but also involved in CS targeting because their individual deletion evoked, in C2C12 myoblasts and C2C12 myotubes, diffuse subcellular distribution and ER-like distribution, respectively (see Table 1). In skeletal muscle fibers, instead, the NH2 terminus exerted a targeting role only in the absence of domain III and domain II did not affect targeting.

Because both front-to-front and back-to-back dimer structures are deemed necessary in recent models of Cu2+-mediated CS oligomerization (30), oligomerization plays a role in CS condensation (11), and oligomerization does not depend on expression of known CS anchoring proteins (11), we argue that...
in the case of CS-HAΔHT, CS-HAΔ24-NH2, and CS-HAΔ2D oligomerization was inhibited, in the absence of endogenous CS, because domains involved in front-to-front or back-to-back dimer formation were absent. As in the case of cardiac CS, oligomerization could be the molecular mechanism sustaining condensation of skeletal muscle CS.

Because CS mRNA is not compartmentalized to the ER of myotubes (22), CS synthesis and translocation could take place anywhere in the endomembrane system and CS exiting from ER might be avoided during SR biogenesis. This scenario is supported by the involvement of the Surf-4 gene during development (13) so that such a pathway might be important only in specific stages of muscle differentiation subsequent to myotube formation. Following this line of reasoning, condensation of CS should be propaedeutic to CS segregation in vivo and to transformation of the ER lumen into a SR-like compartment. Because at early stages of development CS appears already concentrated at discrete sites corresponding to ER tubules or cisternae (38), oligomerization driven by the NH2 terminus and domain II of CS might have physiological relevance.

In skeletal muscle fibers a fully developed SR containing CS is present, so that the condensation ability of the several CS-HA mutants was affected by the presence of endogenous CS. For this reason, CS-HA mutants, unable to engage in either front-to-front or back-to-back interactions, can still form pseudo-oligomers with endogenous CS. On the other hand, only the CS-HA mutant unable to engage in both interactions cannot form both homo- and heterooligomers: CS-HAΔHT cannot conceivably participate in both front-to-front and back-to-back interactions; accordingly, we found that CS targeting was abolished only when both the NH2 terminus and domain III were missing. A plausible interpretation of these results is that oligomerization plays a role in CS targeting to TC and that the NH2 terminus and domain III contain not only target information but also structural information.

In COP II structures, cargo selection for soluble proteins is described by two different models (for a recent review, see Ref. 3): the bulk flow model and the receptor recognition model. In the first model, cargo aggregation and oligomerization in the ER are crucial for both release of soluble cargo from the ER quality control machinery and cargo exclusion from COP II retrograde trafficking, i.e., sequences that promote cargo aggregation or oligomerization must serve as ER exit signals to optimize cargo exit. Intriguingly, it has also been shown that cargo folding and oligomerization are involved in modulation of COP II vesicle formation and ER export competence (2). Because physical protrusion of specialized ER subdomains is mediated by cargo concentration and aggregation (21), CS oligomerization can play an active role in optimizing ER exiting and/or lumen specialization.

The role of oligomerization in the soluble cargo export model might explain the behavior of some of the CS-HA mutants: all the mutants conserve the export-competent sequence but differ in respect to condensation ability; CS-HAΔHT might be excluded from vesicle cargo assembly because of its inability to condense (as inferred from experiments on myoblasts) and to form dimers or higher orders of polymers with endogenous CS (CS-CS-HAΔHT), thus becoming unable to enter COP II vesicles; CS-HAΔ3D can condense, i.e., it is export competent and, as expected, correctly targeted; CS-HAΔ24-NH2 and CS-HAΔ2D are unable to condense in the absence of native CS but are correctly targeted. The apparent discrepancy regarding condensation can be accommodated by two observations: 1) A cardiac CSΔN13 mutant forms large polymers that, even if irregular compared with canonical CS oligomers, could be sufficient for export (30). 2) Both mutants preserve domain III and thus are capable of back-to-back interactions, so that pseudo-oligomers with endogenous CS can be formed and exported from ER. Because deletion of CS domains involved in oligomerization affects targeting in both C1C12 myotubes and muscle fibers and oligomerization is one of the established mechanisms for protein export from ER, a tentative conclusion is that oligomerization is one of the mechanisms relevant for CS targeting in vivo.

In conclusion, data derived from three experimental systems show the coinvolvement of two distinct targeting domains of CS and lead to the plausible conclusion that CS targeting in vivo depends on different molecular mechanisms correlated to the degree of SR differentiation (5). CS targeting may be differently controlled under non-steady-state conditions (SR biogenesis during muscle differentiation) or steady-state conditions (turnover in fully differentiated muscle fibers).

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