Targeting of calsequestrin to sarcoplasmic reticulum after deletions of its acidic carboxy terminus

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Nori, Alessandra, Eleonora Gola, Stefano Tosato, Marcello Cantini, and Pompeo Volpe. Targeting of calsequestrin to sarcoplasmic reticulum after deletions of its acidic carboxy terminus. Am. J. Physiol. 277 (Cell Physiol. 46): C974–C981, 1999.—Calsequestrin (CS) is the Ca$^{2+}$ binding protein of the junctional sarcoplasmic reticulum (jSR) lumen. Recently, a chimeric CS-HA1 obtained by adding the nine-amino-acid viral epitope hemagglutinin (HA1) to the COOH terminus of CS, was shown to be correctly segregated to the sarcoplasmic reticulum [A. Nori, K. A. Nadalini, A. Martini, R. Rizzuto, A. Villa, and P. Volpe. Am. J. Physiol. 272 (Cell Physiol. 41): C1420–C1428, 1997]. A putative targeting mechanism of CS to jSR implies electrostatic interactions between negative charges on CS and positive charges on intraluminal domains of jSR integral proteins, such as triadin and junctin. To test this hypothesis, 2 deletion mutants of chimeric CS were engineered: CS-HA1ΔGlu-Asp, in which the 14 acidic residues [-Glu-(Asp)5-Glu-(Asp)7-] of the COOH-terminal tail were removed, and CS-HA1ΔCOOH, in which the last, mostly acidic, 49 residues of the COOH terminus were removed. Both mutant cDNAs were transiently transfected in HeLa cells, myoblasts of rat skeletal muscle primary cultures, or regenerating soleus muscle fibers of adult rats. The expression and intracellular localization of CS-HA1 mutants were studied by epifluorescence microscopy with use of antibodies against CS or HA1. CS-HA1 mutants were shown to be expressed, sorted, and correctly segregated to jSR. Thus short or long deletions of the COOH-terminal acidic tail do not influence the targeting mechanism of CS.

calcium binding protein; protein targeting

THE SARCOPLASMIC RETICULUM (SR) of skeletal muscle, a network of tubules and cisternae devoted to intracellular Ca$^{2+}$ homeostasis, is composed of two continuous compartments: the nonjunctional SR, enriched in Ca$^{2+}$ pump molecules, and the junctional SR (jSR), juxtaposed to the transverse tubules (TT) and enriched in Ca$^{2+}$ release channels [also known as ryanodine receptors (RyR)] and calsequestrin (CS).

The SR is a subcompartment of the endoplasmic reticulum (ER), as indicated by the coexpression of ER general markers, such as BiP, calnexin, and PDI, with specific molecular components of Ca$^{2+}$ stores, e.g., CS, Ca$^{2+}$ pump, and RyR (26). The molecular differentiation of SR appears to occur from a wide-mesh ER membrane network, to include at an early stage the concentration of CS within membrane-bound structures also containing general ER markers, and progressively to evolve into the establishment of triad couplings between terminal cisternae (TC) and TT (4, 14, 24).

CS is an acidic (3, 22), low-affinity (dissociation constant ∼1 mM), high-capacity (40–50 mol/mol) Ca$^{2+}$ binding protein, the atomic resolution structure of which has been recently described (27). CS segregates to the jSR lumen, where it has been detected as electron-dense material, aggregates, paracrystalline arrays, or fiberlike network (5, 12), and plays a crucial role in the storage of Ca$^{2+}$ between uptake and release cycles (16). CS lacks the characteristic COOH-terminal sequence KDEL (3) that ensures luminal retention, without segregation, to several ER proteins (e.g., BiP and PDI) through continuous recycling from pre-Golgi and Golgi compartments. Although a stage through the Golgi complex seems well established for CS (25), subsequent routes of intracellular trafficking are not yet clear. In particular, it is not known whether CS reaches SR via clathrin-coated cis/medial Golgi-derived vesicles (23) or whether it travels back to the ER, diffuses intraluminally to SR via ER/SR continuities (24, 26), and segregates to the jSR (5, 12). The presence of CS in the ER lumen and nonjunctional SR during early postnatal development (24) as well as in ER subdomains of transfected L6 myoblasts (6) would support the latter possibility.

We have developed a cellular and molecular biology approach to investigate the targeting mechanisms (sorting, retention, and segregation) of CS. A first accomplishment was the characterization of a chimeric cDNA, CS-HA1 cDNA (18): a DNA fragment coding for the nine amino acids of the influenza virus hemagglutinin (HA1) was added at the 3′ terminus of the whole cDNA of rabbit skeletal muscle CS by PCR cloning. The complete construct was transiently transfected in nonmuscle recipient (HeLa) cells, myoblasts isolated from 0- to 3-day-old rat skeletal muscle, and regenerating skeletal muscles of adult rats. Expression and intracellular localization of CS-HA1 were monitored with anti-CS and anti-HA1 antibodies and revealed that 1) chimeric CS-HA1 is correctly sorted to ER/SR compartments in muscle and nonmuscle systems, 2) the HA1 epitope does not alter the structure and/or the sequence of the signal(s) involved in CS retention, 3) the free COOH terminus is not required for targeting of CS, and 4) CS-HA1 segregates to the jSR of regenerating skeletal muscle fibers of adult rats after in vivo transfection of CS-HA1 cDNA (20). Thus CS-HA1 lends itself as a
powerful tool for the identification of targeting sequences of CS by site-directed mutagenesis.

CS segregation to the jSR can be hypothetically accounted for by integral proteins, restricted in their expression to the jSR (8, 11, 13, 29) and able to bind CS [CS-binding protein(s)] with their luminal, mostly basic domain. Triadin (TD) and junctin (J C), both integral membrane proteins, are putative CS-binding proteins and have been recently cloned and sequenced (11, 13); moreover, the RyR can also form complexes with CS (17, 29). CS segregation to the jSR might also be accounted for by specific recognition sites on mostly acidic domains of CS, for interaction with TD and/or J C, and, possibly, with RyR. Given the known primary sequences of CS and of the luminal domains of TD, J C, and RyR, electrostatic interactions are likely. It is not known, however, which and where such putative CS domains might be.

In a first attempt to identify such domains, we have thus engineered two CS-HA1 deletion mutants, CS-HA1 ΔGlu-Asp and CS-HA1 ΔGlu-Asp COOH. In the first case, we have removed the COOH-terminal acidic tail (Glu354, 78 residues at the Asp367) made of 14 amino acids [-Glu-(Asp)5-Glu-] and, in the second case, the 49 residues at the COOH terminus, which are mostly acidic (42% Glu and Asp) (3). The results reported here show that the mostly acidic COOH terminus of CS, irrespective of length of deletion, appears not to be needed for sorting, retention, and segregation of CS to the jSR. The results are also interpreted in the framework of knowledge derived from the crystal structure of CS (27).

**MATERIALS AND METHODS**

**Generation of CS-HA1 ΔGlu-Asp cDNA and CS-HA1 ΔGlu-Asp COOH cDNA**

The CS-HA1 ΔGlu-Asp cDNA was generated as follows: the 948-bp BamHI 1-EcorI 1 cDNA fragment, containing 291 amino acids of rabbit skeletal muscle CS and containing 75 bp of the 5′ untranslated end (3), was isolated from the original plasmid and ligated to the pBSK + vector (Stratagene), excised by BssH II and EcoRI. Modification of the 3′ end, was called pCS-HA1 ΔGlu-Asp COOH.

For the reverse primer, underlined nucleotides represent the coding sequence of the HA1 tag (28) and the stop codon being represented by characters in small capitals. The final construct, devoid of 147 bp at the 3′ end, was called pCS-HA1 ΔGlu-Asp COOH.

Orientation and correct sequence of chimeric mutants were checked by restriction assays, and sequence of the synthetic region was obtained by dideoxy chain termination method (21) with use of modified T7 DNA polymerase.

**Cell Cultures**

HeLa cells. HeLa cells were grown in DMEM containing 2 mM glutamine and 10% FCS.

Primary cultures of skeletal muscle myoblasts and differentiation into myotubes. Primary myoblasts were isolated from hindlimb skeletal muscles of 0- to 3-day-old rats. After the isolated muscles were washed several times in 125 mM PBS, pH 7.4, and subjected to three 20-min stages of trypsinization (2.5% trypsin in PBS) at 37°C and mixing with a vortex every 4 min, supernatants were collected and trypsin was inhibited by addition of 2% horse serum (HS). Cells were then collected by centrifugation and preplated in 9-cm diameter plates for 1 h at 37°C. The myoblast-enriched supernatant was centrifuged, and cells were finally resuspended in DMEM supplemented with 20% FCS and 20 mM glucose, counted, and plated. Differentiation was obtained by changing the medium to DMEM with 10–20% HS and subsequently DMEM with 5% HS. A few nonmuscle cells were occasionally transfected (see Fig. 2D).

Bupivacaine-induced Necrosis and Regeneration of Adult Rat Skeletal Muscle

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 0.4 ml of 0.5% bupivacaine, as described previously (18). Muscles were removed 3 or 10 days later and frozen in liquid nitrogen. In agreement with previous reports (9, 25), the local anesthetic bupivacaine induced almost complete necrosis of the whole soleus by day 3. Regeneration started by day 3 and was completed by day 10.

**Generation of Transient Transfectants**

Twenty-four hours before transfection, HeLa cells or primary myoblasts were seeded onto 25-mm-diameter wells of a 24-well Corning plate containing a 13-mm-diameter round coverslip with a cell density suitable to obtain 50% confluence at the moment of transfection, pCS-HA1 ΔGlu-Asp, pCS-HA1 ΔGlu-Asp COOH, or the control pcDNA3 vector (4 µg/well) was transfected by the calcium phosphate precipitation method (7), as previously described (18). Forty-eight hours after transfection, cells were fixed for immunofluorescence; incubation of myoblasts was prolonged, and the medium was changed for differentiation.

Adult rat soleus muscles were exposed 3 days after bupivacaine injection under ketamine anesthesia and injected with 100 µg of plasmid DNA in 20% sucrose. Rats were killed 7 days later, and transfected and mock-transfected, contralateral muscles were excised, frozen, and processed for immunocytochemistry.

**Immunofluorescence**

Cells were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in PBS for 15 min and permeabilized with 0.3% Triton X-100, 20 mM phosphate buffer, pH 7.4, 0.45 M NaCl,
and 15% goat serum (buffer A) for 30 min. Incubation with polyclonal anti-CS (24, 26) and monoclonal anti-CS (Affinity Bioreagents) or monoclonal and polyclonal anti-HA1 antibodies (BabCO and Santa Cruz Biotechnology, respectively) was performed at room temperature for 1.5 h in buffer A. After they were washed for 1 h, cells were incubated for 30 min with rhodamine isothiocyanate or fluorescein-conjugated anti-mouse or anti-rabbit antibodies (DAKO). Images were obtained with a Zeiss Axioplan microscope or a Leica DMRB microscope. Transverse 9-μm and longitudinal 6-μm sections were obtained for soleus muscles, as described previously (24, 26).

Preparation of Homogenates From HeLa Cells and Myotubes and of SR Vesicles From Rabbit Skeletal Muscles

HeLa cells and myotubes were cultured as described above, transiently transfected with pCS-HA1△Glu-Asp for 2 and 4 days, respectively, harvested in PBS, pH 7.4, rinsed, and lysed in 1 ml of 150 mM NaCl, 15 mM MgCl2, 1 mM EGTA, 1 mM PMSF, 50 mM HEPES, pH 7.5, 10% glycerol, and 1% Triton X-100 for 30 min at 0°C under shaking. The lysate was kept at −20°C until use.

Purified SR vesicles referable to terminal cisternae enriched in CS were prepared as described previously (26) from rabbit skeletal muscles. Protein concentration was determined according to Lowry et al. (15).

SDS-PAGE and Western Blot

SDS-PAGE on 10% acrylamide gels and immunoblot, with anti-CS or anti-HA1 antibodies, were carried out as previously described (19).

Materials

DMEM and complements were purchased from Technogenetics (Milan, Italy), Waymouth’s MB from ICN, and DNA modification and restriction enzymes from Boehringer Mannheim and New England Biolabs, except T7 DNA polymerase, which was purchased from Pharmacia. All other chemicals were obtained from Sigma Chemical.

RESULTS

Construction of the CS-HA1△Glu-Asp cDNA

To obtain a mutant chimeric CS cDNA (CS-HA1△Glu-Asp cDNA), encoding for a CS immunologically distinguishable from endogenous CS and lacking the acidic tail at the COOH terminus, the 3’ end of the coding region of rabbit skeletal muscle CS cDNA was modified by 1) deletion of the last 42 bp coding for 14 amino acids [-Glu-(Asp)5-Glu-(Asp)7-] and 2) addition of a 27-bp fragment coding for 9 amino acids of the influenza virus HA1, as detailed in MATERIALS AND METHODS.

Orientation, sequence, and restriction map of the "synthetic" part of the cDNA were determined by sequencing. Sequence experiments indicate that 1) the synthetic CS cDNA fragment was correctly fused to the EcoR1 site and the whole cDNA was an uninterrupted open reading frame, 2) no additional restriction sites were created by the procedure (e.g., due to partial digestion with EcoR1), 3) the sequence of the CS cDNA corresponded to the original skeletal muscle CS cDNA sequence, and 4) the 3’ end of the CS cDNA had been modified by addition of the tag (HA1) coding sequence and by deletion of the last 14 amino acid residues. The complete cDNA was transferred, by directional cloning, downstream from the CMV promoter of the eucaryotic expression vector pcDNA3, suitable for transient and stable transfection in eucaryotic cells. The resulting construct (called pCS-HA1△Glu-Asp) included the coding regions for the leader sequence, the COOH-terminal-deleted CS, the HA1 tag, and the final stop codon.
Thus the construct was useful for transfection and expression of a mutant chimeric CS, immunologically distinguishable from endogenous CS, and suitable to test the role of the COOH-terminal acidic tail in the segregation mechanism of CS.

**CS-HA1ΔGlu-Asp Expression in Transiently Transfected HeLa Cells: Recognition by Anti-CS and Anti-HA1 Antibodies**

After transfection of HeLa cells, expression of the mutant chimeric CS-HA1ΔGlu-Asp was studied in immunofluorescence experiments with anti-CS antibodies or anti-HA1 antibodies (Fig. 1, A and B, respectively). About 30% of transfected cells were strongly CS positive, and no differences were detected when the reactivity patterns with the two antibodies were compared; thus identification by anti-HA1 monoclonal antibodies was not affected by the overall steric conformation of the mutant protein that could hide the epitope itself. On the contrary, control cells transfected with the empty pcDNA3 vector (mock-transfected cells) were CS negative (Fig. 1C), as expected from the lack of expression of endogenous CS in HeLa cells, nor did they immunostain with anti-HA1 antibodies (Fig. 1D).

The epifluorescence pattern obtained with both antibodies demonstrated that CS-HA1ΔGlu-Asp was retained into the endomembrane network of HeLa cells (Fig. 1, A and B) and did not have a cytoplasmic distribution.

**Compartmentalization of CS-HA1ΔGlu-Asp in SR/ER Membranes of Rat Myotubes in Double-Labeling Experiments**

The possible effects of COOH-terminal deletion were initially studied on transfection of CS-HA1ΔGlu-Asp cDNA in myoblasts. Myoblasts from 0- to 3-day-old rat hindlimb skeletal muscles were cultured in vitro, induced to differentiate into myotubes (18; see MATERIALS AND METHODS), and harvested 4 days after induction. "Transfected myotubes," thus, indicate myotubes obtained from transfected myoblasts by this procedure.

Expression of CS-HA1ΔGlu-Asp was detected in ~20% of rat myotubes by anti-HA1 antibodies, whereas almost all myotubes are CS positive, as indicated by reactivity with anti-CS antibodies. The immunofluorescence pattern was similar with anti-CS antibodies or anti-HA1 antibodies, i.e., fluorescent strands running parallel to the longitudinal axis of the myotubes, suggesting the longitudinal arrangement of the ER/SR membrane network (cf. Ref. 13). Under the prevailing

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Fig. 2. Immunofluorescence of double-labeled rat skeletal muscle myotubes differentiated from myoblasts transiently transfected with CS-HA1ΔGlu-Asp cDNA. Transfection was carried out with pCS-HA1ΔGlu-Asp (A–D) or pcDNA3 (not shown), and fixed myotubes were sequentially decorated with monoclonal anti-CS (A) or polyclonal anti-HA1 (B) antibodies. Distribution of HA1 epitope (red) was detected with anti-HA1 antibodies and rhodamine-conjugated anti-rabbit antibodies; distribution of CS (green) was detected with anti-CS antibodies and fluorescein-conjugated anti-mouse antibodies. C: merge of A and B. Green, multinucleated myotubes in C and D represent nontransfected myotubes expressing endogenous CS. Six different preparations of myotubes were analyzed. Scale bar, 10 µm.
In experimental conditions, discrete CS foci were rarely observed.

Double-labeled transfected myotubes were observed by immunofluorescence: similar patterns of fluorescence were obtained with anti-CS or anti-HA1 antibodies (cf. Fig. 2, A and B). Merge of the two images showed overlap of the antibody reactivity (Fig. 2C); on the contrary, no red regions (corresponding to the HA1 epitope) were observed. Thus transfected myotubes display complete colocalization of recombinant CS-HA1ΔGlu-Asp with endogenous CS, whereas nontransfected myotubes express only endogenous CS (green myotubes in Fig. 2, C and D).

Detection of CS-HA1ΔGlu-Asp in Homogenates Derived From HeLa Cells or Rat Myotubes

The expression of recombinant CS was assayed by Western blots. Transfected HeLa cells or transfected myotubes were cultured as described above, lysed, and analyzed by SDS-PAGE. Western blots of HeLa cell and myotube homogenates with anti-HA1 antibodies show that the pcDNA3-CS-HA1ΔGlu-Asp construct yielded a single protein band with an apparent molecular weight of ~63,000 (Fig. 3, lanes b–f), comparable to that of the CS isoform from rabbit skeletal muscle SR (Fig. 3, top band, CSsk, in lanes c and g). The deduced molecular weight of the mutant CS (41,776) is, in fact, very close to that of skeletal muscle CS (41,630) (3), since the net difference is given by five amino acids. SR vesicles (Fig. 3, bottom band, CSc, in lanes c and g) and myotubes (Fig. 3, bottom band, CSc, in lane h) display, as expected, the minor cardiac CS isoform (1, 22, 26).

The results also indicate that the epitope(s) recognized by either antibody was within the recombinant protein. Moreover, no proteolytic breakdown products could be detected, and this rules out the possibility that the chimeric protein, as it may happen (6, 20), undergoes accelerated or altered turnover, which, in turn, may result in misleading interpretation of immunofluorescence data.

Targeting of CS-HA1ΔGlu-Asp in Regenerating Skeletal Muscle Fibers of Adult Rats

The last experimental approach relies on knowledge that 1) bupivacaine injected into the soleus muscle of adult rats (9) causes complete necrosis within 3 days and regeneration in the following 7 days and 2) regenerating muscle fibers display a higher efficiency of transfection (25). Thus transfection of CS-HA1ΔGlu-Asp cDNA in soleus muscle allows us to determine whether and where the mutant chimeric CS-HA1ΔGlu-Asp is targeted in vivo, in particular, whether it segregates to the jSR, at the completion of the regeneration process (18).

Figure 4 shows, 10 days after bupivacaine treatment, all skeletal muscle fibers were labeled with...
anti-CS antibodies (Fig. 4A), whereas only a few fibers, as expected, were labeled with anti-HA1 antibodies (Fig. 4B), as judged, in both cases, by immunofluorescence of transverse sections. Thus a few fibers express the recombinant CS-HA1ΔGlu-Asp.

Localization of CS-HA1ΔGlu-Asp was thoroughly investigated by immunofluorescence of double-labeled longitudinal sections of soleus muscle fibers. Figure 5, A (anti-CS antibodies) and B (anti-HA1 antibodies), shows that CS-HA1ΔGlu-Asp was indeed localized at the A-I interface, as indicated by the typical, regular banding pattern of punctate fluorescence, i.e., two rows of triads on either side of the Z line. Merge images (Fig. 5C) clearly indicate colocalization of endogenous and recombinant CS-HA1ΔGlu-Asp at the TC level.

Targeting of CS-HA1Δ49COOH in Regenerating Skeletal Muscle Fibers of Adult Rats

Glu and Asp residues are clustered along the last 49 amino acids of the CS COOH terminus and make up 42% of it (3). To further examine the role of negatively charged residues at the COOH terminus in the segregation mechanism of CS, we next studied expression and subcellular localization of a second deletion mutant, CS-HA1Δ49COOH. In preliminary experiments, CS-HA1Δ49COOH cDNA was transfected in HeLa cells, and expression of mutant CS-HA1 was studied by immunofluorescence with anti-CS antibodies or anti-HA1 antibodies; ~30% of transfected cells were strongly CS positive, and no differences were detected in the reactivity patterns of the two antibodies (not shown).

Localization of CS-HA1Δ49COOH was investigated by immunofluorescence of double-labeled longitudinal sections of regenerating and transfected soleus muscle fibers. Figure 6, A (anti-CS antibodies) and B (anti-HA1 antibodies), clearly shows that CS-HA1Δ49COOH displayed a regular pattern of punctate fluorescence, as implied by two contiguous bands of punctate labeling localized at the A-I interface.

DISCUSSION

Targeting of CS includes sorting, retention to the SR, and segregation to the jSR of skeletal muscle. The rationale of experiments reported here is that by comparing the intracellular routing and subcellular localization of wild, chimeric, or mutant/chimeric CSs that can be secreted, retained but not segregated, or retained and segregated to restricted membrane compartments, i.e., jSR, information is gathered regarding the intrinsic targeting mechanism(s) of CS.
Two deletion mutant cDNA clones, CS-HA1ΔGlu-Asp
and CS-HA1Δ49COOH, have been designed and charac-
terized to verify one of the putative targeting mecha-
nisms of CS to the jSR; such a mechanism would
involve electrostatic interactions between acidic do-
 mains of CS and basic luminal domains of jSR integral
proteins, e.g., TD, J C, and possibly RyR. The two
deletion mutants differ in the extent of deletion at the
COOH terminus, 14 vs. 49 amino acid residues.

The first recombinant CS, CS-HA1ΔGlu-Asp, was
engineered so that 1) intracellular routing and subcel-
lar localization could be monitored by specific antibod-
ies directed to the chimeric tag HA1 and 2) a 42-bp
fragment was deleted at the 3' end of the coding region
to investigate the specific role of the COOH-terminal
tail, made of 14 acidic amino acids [-Glu-(Asp)5-Glu-
(Asp)7-], in the electrostatic interaction(s) with intralu-
minal basic domains of jSR proteins.

The present results show that the mutant CS-
HA1ΔGlu-Asp is 1) sorted to endomembrane compart-
ments in HeLa cells (Fig. 1), 2) sorted and retained to
the SR of differentiating rat myotubes (Fig. 2), and 3)
segregated to the SR of skeletal muscle fibers (Fig. 5)
after in vivo transfection of the recombinant cDNA.

Clear-cut data are derived from in vivo transfection
of CS-HA1ΔGlu-Asp cDNA into regenerating skeletal
muscle fibers of adult rats (Figs. 4 and 5); mutant CS
is not only sorted and retained to the SR but, more
importantly, also segregates to TC. Expression of CS-
HA1ΔGlu-Asp during muscle regeneration, i.e., during
SR biogenesis, TC development, and triad formation,
indicates that deletion of the COOH-terminal acidic
tail does not affect CS segregation to the jSR. Thus not
only is the free COOH terminus of CS not required for
sorting, retention, and segregation to the jSR (18), but
also the COOH-terminal acidic tail is not, apparently,
involved in protein-protein electrostatic interactions.

Franzini-Armstrong et al. (5), on the basis of freeze-
fracture studies of skeletal muscle triads, described a
network of CS aggregates in the jSR lumen as being
constituted by long fiber structures, which are fully
compatible with the Ca2+-induced linear polymers or
multimer of dimers of CS recently described by Wang
et al. (27). The present results indicate that deletion of
the COOH tail does not appear to interfere with dimeriza-
tion and polymerization of CS, in agreement with crystal
structure data (27).

If multiple protein-protein interactions were envi-
ioned to account for CS targeting and if the CS COOH
terminus were indeed involved, one alternative interpre-
tation of our results would be that CS-HA1ΔGlu-Asp
segregates, despite the “short” CS tail deletion. Be-
cause the last 49 amino acid residues of the COOH
terminus are mostly acidic, i.e., 42% Glu-Asp (3), it
might be that the short deletion is not sufficient to
abolish the interaction of CS with the anchoring pro-
tein(s). To test the effects of a longer deletion at the
COOH terminus, a second deletion mutant CS cDNA,
CS-HA1Δ49COOH, was developed and transfected in
vivo into regenerating skeletal muscle fibers of adult
rats. Because CS-HA1Δ49COOH is segregated to TC (Fig.
6), it might be safely concluded that the acid COOH
terminus, as a whole, does not affect CS targeting.

Segregation of CS to jSR may be a complex mecha-
nism and entail docking, i.e., heterologous protein-
protein interactions, and self-aggregation of CS, i.e.,
homologous protein-protein interactions. Intra- and/or
intermolecular interactions between the SAH and DBH
sites on CS have been proposed as crucial for Ca2+-
induced folding (10) and self-aggregation (10, 27). If we
assume, despite evidence to the contrary (present
results and Ref. 27), that the COOH-terminal acidic tail
were essential for segregation to the jSR, the presence
of endogenous CS (wtCS) in myotubes and regenerat-
ing muscle fibers (Figs. 2, 5, and 6) might lead to
erroneous interpretations. Mixed (CS-HA1ΔGlu-Asp/
wtCS or CS-HA1Δ49COOH/wtCS) aggregates could still
form, whereas the acidic tails of wtCS might interact
with jSR anchoring proteins. In vitro and in vivo
experimental systems are being developed to address
directly the latter issue.

Overall, the present results are in agreement with
recent crystal structure data (27): Ca2+-induced CS
dimerization interface forms a large pocket lined with
many acidic residues. This disordered C-terminal seg-
ments (residues 352–367) “create a very electronega-
tive enclosure within this pocket.” Thus it appears that
the mostly acidic COOH terminus, on the basis of not
only present data with two different deletion mutants
but also distinct structural considerations (27), is not
involved in homologous and heterologous protein-
protein interaction but in Ca2+ binding.

Finally, we note that the targeting mechanism of CS,
based on electrostatic interactions, may still hold true:
it is entirely plausible that other CS domains are
involved, and among them are to be listed acidic
stretches located on the surface of each of the three
topological domains (2, 27), the switch points of domain
II and III (around residues 228–229), rich in acidic
amino acids (27), and the NH2 terminus of CS (8,
27, 29).

We thank Dr. F. Patiri for help in some immunofluorescence
experiments.

This work was supported by Telethon, Italy, Grant 669 and by
funds from the Ministero dell’Università e della Ricerca Scientifica e
Tecnologica (1997, Programma di ricerca di rilevante interesse
nazionale on “Biopatologia della fibra muscolare scheletrica”).

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Received 28 December 1998; accepted in final form 16 July 1999.

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