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

ALESSANDRA NORI, 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\textsuperscript{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-HA1\textsubscript{ΔGlu-Asp}, in which the 14 acidic residues [Glu(Asp)-Glu(Asp)-] of the COOH-terminal tail were removed, and CS-HA1\textsubscript{ΔCOOH}, 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.

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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-HA1ΔGlu-Asp and CS-HA1Δ49COOH. In the first case, we have removed the COOH-terminal acidic tail (Glu354-[Asp]-) made of 14 amino acids [-Glu-(Asp) 5-Glu-] (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-HA1ΔGlu-Asp cDNA and CS-HA1Δ49COOH cDNA

The CS-HA1ΔGlu-Asp cDNA was generated as follows: the 948-bp BamH I-EcoR I cDNA fragment, encoding 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) pre-cleaved with BamH I and EcoR I. Modification of the 3' cDNA end with deletion of the last 42 bp and addition of 27 bases coding for 9 amino acids of HA1 (3) was performed by PCR. The following PCR primers were used: 1) forward primer consisting of 5'-GAATTCTTAGAGATCTCAAGTCT-3' and 2) reverse primer consisting of 5'-CTAGGCTAGCATATTGTTGTGTGTATCCAGCAC-3'. For the reverse primer, underlined nucleotides represent the coding sequence of the HA1 tag (28), whereas the stop codon is indicated by characters in small capitals. The final construct, devoid of 147 bp at the 3' end, was called pCS-HA1Δ49COOH.

Orientation and correct sequence of chimeric mutants were checked by restriction assays, and sequence of the synthetic region was obtained by the 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 rat 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 pre-plated in 9-mm-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 2% HS. A few nonmuscle cells were occasionally transplanted (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-HA1ΔGlu-Asp, pCS-HA1Δ49COOH, 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\D 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\D Glu-Asp cDNA

To obtain a mutant chimeric CS cDNA (CS-HA1\D 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.

Fig. 1. Immunofluorescence pattern of HeLa cells transiently transfected with pCS-HA1\D Glu-Asp cDNA. Two days after transfection with pCS-HA1\D Glu-Asp (A and B) or pcDNA3 (C and D), cells were fixed and decorated with monoclonal anti-HA1 (B and D) or polyclonal anti-CS (A and C) antibodies. Eight different preparations of HeLa cells were analyzed. HA, hemagglutinin; CS, calsequestrin. Scale bar, 5 µm.
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-HA1Glu-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-HA1Glu-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-HA1Glu-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-HA1Glu-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-HA1Glu-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-HA1Glu-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 conditions of the experiment, the COOH-terminal deletion of 9 amino acids did not affect the correct targeting of CS-HA1Glu-Asp to the endomembrane network, but it did prevent recognition by anti-CS antibodies.
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 that, 10 days after bupivacaine treatment, all skeletal muscle fibers were labeled with 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 pCS-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, CS Sk, 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, CS C, in lanes c and g) and myotubes (Fig. 3, bottom band, CS C, 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.
localization of CS-HA1\textsuperscript{D}Glu-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-HA1\textsuperscript{D}Glu-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-HA1\textsuperscript{D}Glu-Asp at the TC level.

Targeting of CS-HA1\textsuperscript{D}49\textsubscript{COOH} 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-HA1\textsuperscript{D}49\textsubscript{COOH}. In preliminary experiments, CS-HA1\textsuperscript{D}49\textsubscript{COOH} cDNA was transfected in HeLa cells, and expression of mutant CS-HA1 was studied by immunofluorescence with anti-CS antibodies or anti-HA1 antibodies; \(\sim 30\%\) 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-HA1\textsuperscript{D}49\textsubscript{COOH} 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-HA1\textsuperscript{D}49\textsubscript{COOH} 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 characterized to verify one of the putative targeting mechanisms of CS to the jSR; such a mechanism would involve electrostatic interactions between acidic domains of CS and basic luminal domains of jSR integral proteins, e.g., TD, JC, 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 subcellular localization could be monitored by specific antibodies 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 intraluminal basic domains of jSR proteins.

The present results show that the mutant CS-HA1ΔGlu-Asp is 1) sorted to endomembrane compartments in HeLa cells (Fig. 1), 2) sorted and retained to the SR of differentiating rat myotubes (Fig. 2), and 3) segregated to the jSR 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 segregated to the jSR of skeletal muscle fibers (Figs. 2, 5, and 6) after in vivo transfection of the recombinant cDNA.

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Address for reprint requests and other correspondence: P. Volpe, Dept. di Scienze Biomediche Sperimentali, Universita` degli Studi di Padova, viale G. Colombo 3, 35121 Padua, Italy (E-mail: volpe@civ.bio.unipd.it).

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