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Newly Synthesized Calsequestrin, Destined for the Sarcoplasmic Reticulum, Is Contained in Early/Intermediate Golgi-derived Clathrin-coated Vesicles*

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Kathleen Thomas,[a] Javier Navarro,[b,c] Robin J. J. Benson,[d] Kevin P. Campbell,[e] Richard L. Rotundo,[f] and Richard E. Fine[g,h,i]

From the Departments of [a]Microbiology, [b]Physiology, [d]Anatomy, and [g]Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118; the [h]Eleanor Nourse Rogers Memorial Veterans Administration Hospital, Bedford, Massachusetts 01730; [e]Department of Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52242; and the [f]Department of Anatomy and Cell Biology, University of Miami School of Medicine, Miami, Florida 33101

We have examined the possible role of clathrin-coated vesicles (CVs) in the genesis of the sarcoplasmic reticulum (SR) in developing chick skeletal myotubes. Calsequestrin (CSQ) a luminal Ca^{2+} binding protein of the terminal SR cisternae, is contained within the vesicle lumen of skeletal muscle CVs in substantial amounts, approximately four molecules/CV. Employing 3-day cultures of chick skeletal myotubes we demonstrate that after a 30-min labeling with [^{35}S]methionine and cysteine, radioactivity in CSQ remains high in the CVs 45 min later and then declines, while labeled CSQ in the SR continues to rise. No CSQ appears to be secreted. All of the CSQ in both the CVs and SR is sensitive to the activity of endoglycosidase H, and a significant fraction also binds to wheat germ agglutinin. Based on these results, we discuss the hypothesis that a selective CV-mediated pathway exists in developing skeletal muscle cells for the transport of CSQ from the early/intermediate Golgi apparatus to the SR.

The sarcoplasmic reticulum (SR)[1] is the major intracellular calcium sequestering organelle in skeletal and heart muscle. The development of the vertebrate muscle SR is still not well understood. The three major proteins which have been shown to be present in the SR are all synthesized and inserted into the rough endoplasmic reticulum (reviewed in MacLennan and Campbell, 1979; Martonosi, 1982). The Ca^{2+} ATPase is not glycosylated. The M_r 55,000 integral membrane glycoprotein is a high mannose containing glycoprotein, while calsequestrin (CSQ) is a soluble, *N*-linked carbohydrate containing species.

In perhaps the most interesting study of this organelle's development, MacLennan and associates demonstrated that CSQ immunoreactivity is detected in developing cultures of rat myotubes at least 10 h before any Ca^{2+} ATPase staining is visualized (Jorgensen *et al.*, 1977). Initially CSQ staining is seen in the perinuclear region of the cell. At later times, corresponding to the first appearance of Ca^{2+}-ATPase staining, CSQ staining occupies progressively larger regions adjacent to the nuclei and takes on a fibrous appearance. In contrast, Ca^{2+}-ATPase staining appears diffused throughout the cytoplasm. These findings suggest that the Ca^{2+}-ATPase and CSQ may follow different routes after their synthesis before reaching the SR.

More recent electron microscopic immunocytochemical work by Jorgensen and colleagues has provided evidence, in adult muscle, that the Ca^{2+}-ATPase is found throughout the SR while CSQ is confined to the terminal cisternae underlying the T tubules (Jorgensen *et al.*, 1983). Recently, both proteins have been cloned (MacLennan *et al.*, 1985a; Fliegel *et al.*, 1987). CSQ has been found to have only one *N*-linked glycosylation site containing only mannose and *N*-acetylglucosamine in a 3:2 ratio (Jorgensen *et al.*, 1977).

In this report we present data consistent with the hypothesis that a quantitatively very significant and selective pathway for delivery of CSQ from the early/intermediate Golgi to the SR is mediated by clathrin-coated vesicles (CVs). We discuss the significance of this pathway with respect to SR biogenesis.

MATERIALS AND METHODS

11- or 17-day-old chicken embryos were obtained from Spafas, Inc. (Norwich, CT), ^{125}I-protein A-Sepharose and endoglycosidase H from Du Pont-New England Nuclear. Protein A-Sepharose beads were obtained from Bio-Rad.

Tran[^{35}S]label, 80% methionine, 20% cysteine (1000–1200 mCi/mmol) was purchased from ICN Radiochemicals. Zeta-bind was purchased from AMF-Cuno (Meriden, CT). WGA-Sepharose was from E. Y. Laboratories (San Mateo, CA), and ConA-Sepharose was obtained from Sigma. A polyclonal antibody against chick heart CSQ was provided by Dr. Kevin P. Campbell (University of Toronto, Ontario, Canada). A monoclonal antibody against rabbit skeletal Ca^{2+}-ATPase was provided by Dr. David H. MacLennan.

Isolation of Calsequestrin—The method of Cala and Jones (1983) was followed to purify calsequestrin from adult chicken sarcoplasmic reticulum.

Western Blots of Calsequestrin and Ca^{2+}-ATPase—Proteins from SDS gels were transferred to Zeta-bind according to the method of Gershoni and Palade (1982). The blots were incubated for 2 h with a

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[i] To whom correspondence should be addressed: Dept. of Biochemistry, Boston University School of Medicine, 80 E. Concord St., Rm. K-402, Boston, MA 02118.

[1] The abbreviations used are: SR, sarcoplasmic reticulum; AChE, acetylcholinesterase; CSQ, calsequestrin; CV, clathrin-coated vesicles; ConA, concanavalin A; endo H, endoglycosidase H; WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

1:1000 dilution of anti-CSQ at 37 °C and then washed for 1 h with Triton X-100 and deoxycholate. ^{125}I-Protein A (1,000,000 cpm) was added for 2 h at 37 °C, and then the blots were washed overnight at room temperature. Anti-mouse IgG conjugated to alkaline phosphatase (Sigma) was used as the second antibody, with the monoclonal anti-Ca^{2+}-ATPase.

Metabolic Labeling of Myotube Cultures with [^{35}S]Methionine/Cysteine—Chick embryo muscle cultures were prepared on 100-mm culture dishes or on 24 × 24-cm plates as described previously (Benson *et al.*, 1985). At 3 days, cultures were rinsed once with phosphate-buffered saline and incubated for various times in methionine-free Eagle's medium containing 10% horse serum and 50–100 μCi/ml [^{35}S] methionine/cysteine. For pulse chase experiments, cells were labeled for 30 min, rinsed once, and incubated for increasing times up to 150 min with chase medium containing a 1000-fold excess of unlabeled methionine.

CV Preparation—CVs were isolated from either 17-day-old chick embryo skeletal muscle or from 3–5 day cultures of chick skeletal myotubes according to the methods of Benson *et al.* (1985). The preparations were at least 95% CVs by the criterion of negative stain electron microscopy.

Preparation of Sarcoplasmic Reticulum Vesicles—Fractionation of adult or embryonic skeletal muscle and cultured muscle myotubes was performed according to a modification of the method of Jones *et al.* (1979). Briefly, the muscle or myotubes were homogenized in a Polytron 3 times for 30 s each in phosphate-buffered saline (pH 7.2) and then spun at 15,000 × *g* to remove mitochondria and nuclei. The pellet was reextracted, and then the supernatants were combined and incubated with 0.6 M KCl for 1 h. Next, the supernatants were pelleted for 45 min at 150,000 × *g*. The pellet was resuspended and incubated with 50 mM histidine, 15 mM $CaCl_2$, 5 mM Tris oxalate, 16 mM Tris/EGTA, 30 mM $MgCl_2$, 30 mM ATP, and 100 mM KCl (pH 6.8) for 30 min at room temperature. At 15 min of incubation, additional calcium oxalate and ATP were added. The solution was loaded directly onto a discontinuous sucrose gradient consisting of 1.5, 1.0, 0.8, and 0.6 M sucrose plus 0.3 M KCl, 0.05 M sodium pyrophosphate, and 0.1 M Tris (pH 7.0). The gradients were spun for 2 h at 27,000 rpm in a Beckman SW-27 rotor. Supernatants from cultured myotubes yielded six fractions, including a pellet, which were collected and frozen in 10 mM Hepes, 0.25 M sucrose (pH 7.0) for future use. Adult rooster muscle yielded only three separate fractions.

Immunoprecipitation of Calsequestrin and Subsequent Treatment by Endo H—Coated vesicles or sarcoplasmic reticulum vesicles were solubilized with 0.5% Triton X-100, 0.5% SDS, 0.25% deoxycholate, and 0.25% bovine serum albumin (immunomix). 10 μl of polyclonal anti-calsequestrin antibody or normal rabbit serum was added to each supernatant and incubated overnight at 4 °C. Next, 50 μl of protein A-agarose beads were added to each supernatant and rotated for 2 h at room temperature and washed 4 times with immunomix and 1 time with 100 mM sodium citrate buffer (pH 5.5). 74 ng of endoglycosidase H was added to the beads in 300 μl of sodium citrate buffer and incubated for 2 h at 37 °C. The reaction was stopped by addition of Laemmli sample buffer.

RESULTS

Calsequestrin Is Contained in a Membrane Protected Form within CVs Isolated from 17-Day-old Chick Embryo Skeletal Muscle—We have previously demonstrated that CVs from 17-day-old embryonic skeletal muscle can be purified by sucrose density gradients to better than 95% homogeneity as determined by both negative staining and thin sectioning of CV preparations (Benson *et al.*, 1985). The CVs can then be purified to apparent homogeneity by agarose gel electrophoresis (Rubenstein *et al.*, 1981; and Porter-Jordan *et al.*, 1986). Fig. 1*A* demonstrates that CVs purified to virtual homogeneity on agarose gels contain CSQ as defined by its apparent molecular weight (58,000) and reaction with a monospecific antibody prepared against muscle CSQ (Jorgensen *et al.*, 1983). Fig. 1*B* demonstrates that when an identical aliquot is treated with 2 M urea to remove clathrin and associated coat proteins (Blitz *et al.*, 1977) the resulting smooth vesicles remain very near the origin. All the detectable CSQ now also is found at the origin, indicating its association with the membranes of CVs. A protein band corresponding to CSQ

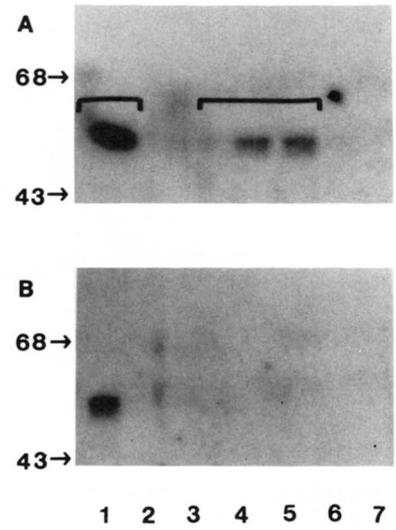


FIG. 1. **Calsequestrin is associated with chick embryo skeletal muscle coated vesicles purified to near homogeneity by agarose gel electrophoresis.** *A*, 50 μg of sucrose gradient-purified coated vesicles were subjected to agarose gel electrophoresis as described (Rubenstein *et al.*, 1981). Seven sequential 0.5-cm fractions were excised from each lane and lyophilized. Each fraction was resuspended in Laemmli sample buffer and subjected to SDS-PAGE. An immunoblot using anti-CSQ was performed to detect the location of CSQ in the agarose gel. The numbers *1–7* represent lanes of the SDS gel corresponding to the seven sequential segments of the agarose gel. *1* represents the origin and *7* represents the most anodal segment. The *two bars* indicate the fractions which contain clathrin detectable by Coomassie Blue staining. *Numbers* to the *left* of this and subsequent figures refer to the molecular mass in kilodaltons of the marker proteins. *B*, To an identical 50-μg aliquot of CVs was added solid urea to a final concentration of 2 M. The solution was then centrifuged at 100,000 × *g* for 30 min in a Beckman Airfuge. The pellet, which contained uncoated vesicles as ascertained by negative staining electron microscopy, was resuspended in the original volume and subjected to agarose gel electrophoresis in an adjacent well to the CV sample in *A*. All subsequent steps were identical to those described in *A*. The *bar* shows the location of the uncoated vesicles as determined by negative stain electron microscopy.

can be visualized in Coomassie Blue or silver-stained preparations of CVs and has a characteristic yellow color in silver-stained preparations (data not shown). Under similar conditions the Ca^{2+}-ATPase which is more abundant then CSQ in the SR cannot be detected in CVs using a monoclonal antibody directed against the Ca^{2+}-ATPase, even at lengthy film exposure (data not shown).

To determine whether CSQ was contained within the CV membrane in a latent form, we treated CVs with trypsin in the presence and absence of detergent. As can be seen in Fig. 2, CSQ is totally resistant to the action of trypsin in the absence of detergent but is degraded to one detectable smaller fragment in its presence. Two minor lower molecular weight peptide fragments of CSQ are also present in the CVs (Fig. 2, *lanes 1 and 3*) which are also degraded by trypsin only in the presence of detergent. 2 M urea treatment also does not remove CSQ from the resulting smooth vesicles (Fig. 2*e*). These data indicate that CSQ is contained within the membrane vesicles of CVs.

Using the Western blotting technique, we have determined a standard curve for CSQ concentration using CSQ purified as described previously (Cala and Jones, 1983) from chicken breast muscle SR. Fig. 3 shows the results and a comparison with the signal obtained when 50 μg of chick muscle CVs were subjected to the same procedure. Densitometry of the autoradiogram revealed that there were approximately 4.0 μg of

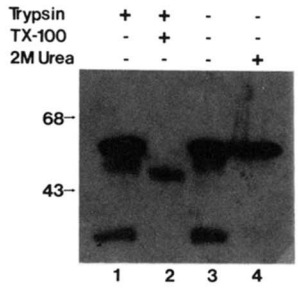
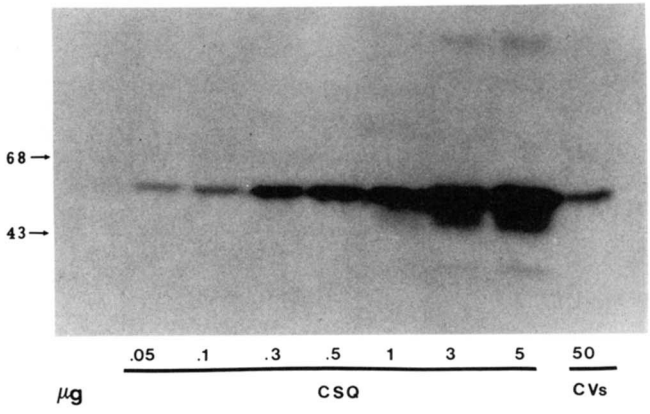


FIG. 2. **Calsequestrin is contained within the coated vesicle lumen.** Coated vesicles were treated with 0.025% trypsin alone (*lane 1*) or trypsin plus 0.3% Triton X-100 (*TX-100*) (*lane 2*) for 1 h at 37 °C. At the end of the incubation, each tube received 0.1% soybean trypsin inhibitor. Sample number 3 (*lane 3*) contained non-treated CVs. Sample number 4 (*lane 4*) received 2 M urea, Tris-HCl, pH 8.2, to remove the clathrin coat. After 2 h at room temperature, the vesicles were pelleted. All samples were subjected to SDS-PAGE and then an immunoblot was performed to detect CSQ.



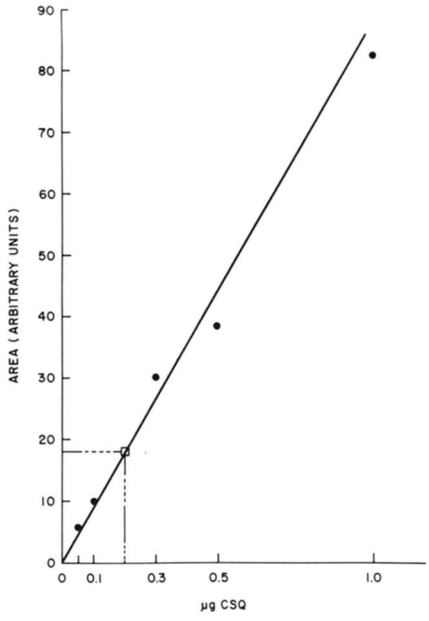


FIG. 3. **The quantity of calsequestrin in coated vesicles as determined by Western blot analysis.** Calsequestrin was purified by the method of Cala and Jones (1983). 50 μg of coated vesicles were run on SDS-PAGE with varying amounts of purified calsequestrin. A densitometric scan of the immunoblot yielded a standard curve from which the amount of calsequestrin in coated vesicles was determined. *A*, Western blot. *B*, standard curve of CSQ concentration.

CSQ/mg CVs. Assuming that clathrin makes up approximately 40% of the mass of the CV (Steven *et al.*, 1983) and that there are approximately 100 molecules of clathrin/CV (Crowther *et al.*, 1976), there are approximately 4 molecules of CSQ present/CV. Using the same technique for determining the amount of CSQ we also determined that there are approximately 0.5 μg of CSQ present/mg protein in 3-day-old cultures of developing chick skeletal muscle cells.

Some CSQ Molecules Are Contained in CVs Which Also Contain Acetylcholinesterase (AChE)—We have recently described an AChE-mediated density shift technique which we have utilized to separate CVs which contain AChE from those which do not (Porter-Jordan *et al.*, 1986). Using this technique we have previously demonstrated that essentially all the AChE contained in developing muscle CVs is newly synthesized and that CVs containing AChE can be operationally defined as "exocytic" (Benson *et al.*, 1985; Porter-Jordan *et al.*, 1986). In order to determine whether CSQ is also contained in exocytic CVs, we subjected CVs to the AChE-mediated density shift technique and determined the amount of CSQ and AChE shifted (Table I). We were able to shift approximately 30% of the total AChE and 15% of the CSQ molecules by this procedure. This finding indicates that while some CVs contain both proteins, there may be a class of CVs which contains CSQ but not AChE, as well as a class which only contain AChE.

The Kinetics of the Appearance of [^{35}S]Methionine/Cysteine-labeled CSQ in CVs and SR Are Consistent with the CV Being an Intermediate in CSQ Transport between ER/Golgi and SR—In order to determine the origin and the destination of CSQ containing CVs we employed cultures of chick embryo skeletal myotubes as described previously (Benson *et al.*, 1985). We also described previously the preparation of highly purified CVs from these cultures (Benson *et al.*, 1985). CSQ is associated with these tissue culture derived CVs as demonstrated by its having the identical mobility as CVs and clathrin on agarose gels (data not shown).

We also employed a modification of the Ca^{2+} oxalate-mediated density shift (Jones *et al.*, 1979) to isolate a highly enriched fraction of SR from tissue-cultured myotubes as described under "Materials and Methods." The two densest fractions consist of enclosed membrane vesicles and are enriched in markers for ER and de-enriched for markers for Golgi, lysosomes, and plasma membranes (data not shown). Fig. 4 shows Western blots of these gradients using anti-Ca^{2+}-ATPase and CSQ sera, respectively. As can be seen, the

TABLE I

A CV population which contains AChE also contains CSQ

Two identical aliquots (200 μg) of CVs were incubated with the Karnovsky-Roots reagents plus (A) or minus (B) acetylthiocholine and subjected to the sucrose density shift protocol as described previously (Porter-Jordan *et al.*, 1986). Four equivalent fractions were collected from each gradient; fraction 1 represents the least dense, fraction 4 the most dense. A 0.05-ml aliquot of each fraction was assayed for AChE activity by the method of Johnson and Russell (1977). A 200-μl aliquot of each fraction was subjected to SDS-PAGE and immunoblotting with anti-CSQ. The amount of CSQ in each sample was determined by densitometry. The AChE and CSQ results are expressed as percentages of the totals on each gradient.

	A. (+) Acetylthiocholine		B. (−) Acetylthiocholine	
	AChE	CSQ	AChE	CSQ
	% total			
Fraction 1	16	18	44	33
Fraction 2	36	52	45	61
Fraction 3	17	5	11	6
Fraction 4	31	15	0	0

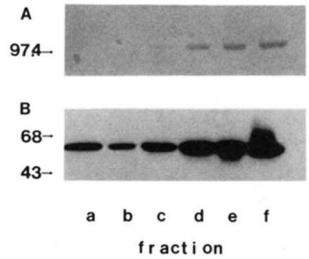


FIG. 4. **Characterization of sarcoplasmic reticulum vesicles.** SR was prepared as described under "Materials and Methods." Six fractions were collected sequentially from the discontinuous sucrose gradient. *Lane a* represents the least dense fraction, *lane f* the densest. 100 μg of each fraction were run on SDS-PAGE. An immunoblot using the polyclonal CSQ antibody was performed with ^{125}I-protein A as the second antibody. A monoclonal antibody plus an alkaline phosphatase conjugated anti-mouse IgG was used to detect the Ca^{2+}-ATPase. *A*, immunoblot using anti-Ca^{2+}-ATPase. *B*, immunoblot using anti-CSQ.

densest two fractions (*e* and *f*) are highly enriched in the Ca^{2+}-ATPase as well as CSQ. Control incubations with either ATP or oxalate absent, gave no CSQ or Ca^{2+}-ATPase staining in fractions *e* or *f* (data not shown). Fraction *f* was designated as highly enriched SR in subsequent experiments.

One apparent difference between the distributions of Ca^{2+}-ATPase and CSQ was noted. There was no detectable Ca^{2+}-ATPase in the lightest fractions of the gradients even at lengthy film exposures. In contrast, a second peak of CSQ was present in fraction *a*, which corresponded to the peak activity of [^{3}H]PN200-110, a dyhydropyridine which is a marker for T tubules (Navarro, 1987).

We next determined the kinetics of appearance of [^{35}S] methionine/cysteine-labeled CSQ in isolated CVs and SR fractions purified from 3-day-old cultures of myotubes. As shown in Fig. 5, the results demonstrate that after a 30-min labeling, 2,500 cpm of immunoprecipitated CSQ is present in isolated CVs. After a 45-min chase the amount of counts/min is about the same, while by 90 min there is a small decrease. By 120 min of chase no labeled CSQ can be detected in CVs. In contrast, the amount of labeled CSQ detected in the SR rises significantly during the 90-min chase period. These data are consistent with the hypothesis that CVs are intermediates at one or more stages of CSQ transport between the rough endoplasmic reticulum and the SR.

To more precisely define the stage(s) of CV-mediated transport, we treated anti-CSQ derived immunoprecipitates from radiolabeled CVs at 30 min and 30 min plus 45 min chase with endoglycosidase H (endo H), which only cleaves *N*-linked oligosaccharides containing 5 or more mannose residues (Hubbard and Ivatt, 1981). Fig. 6, *C* and *D*, shows the results. As can be seen, both CV samples are totally sensitive to endo H as shown by an approximate decrease of 2000 Da, consistent with the removal of 7–9 carbohydrate residues. Fig. 6*E* demonstrates that all of the CSQ accumulated in the SR during a 10-h labeling also remains endo H-sensitive. We also investigated the endo H sensitivity of total cell-associated CSQ. As can be seen in Fig. 6*B*, all detectable cell-associated CSQ is endo H-sensitive.

We also labeled cells with [^{35}S]methionine/cysteine for 10 h to see if any CSQ was secreted. We found no detectable secreted CSQ (Fig. 6*A*) while 18,000 cpm of CSQ was made by the cells during this time and retained inside the cells, in an endo H-sensitive form (data not shown).

CSQ Is Partially Wheat Germ-Agglutinatable in Both CVs and SR—As reported previously (Rotundo, 1984) skeletal muscle AChE becomes wheat germ-agglutinatable 1 h before

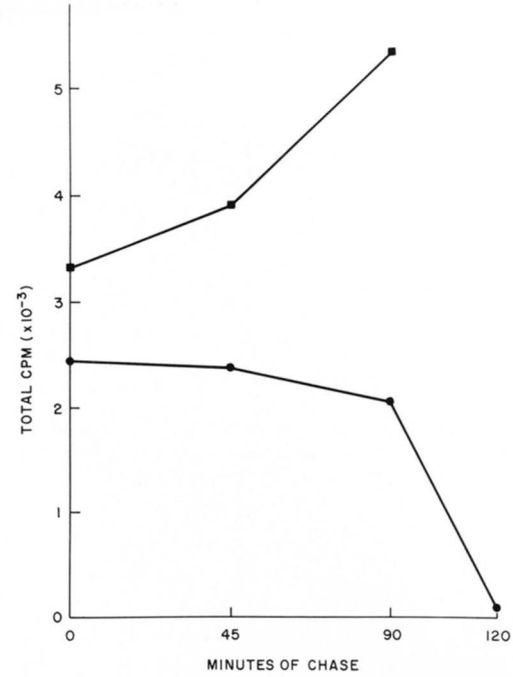


FIG. 5. **Labeled calsequestrin exits coated vesicles and enters the SR over time.** Cultured chick myotubes were labeled for 30 min with [^{35}S]methionine/cysteine and then chased for varying amounts of time. Cells were collected and fractionated. Coated vesicles (●) and sarcoplasmic reticulum (■) were prepared as described under "Materials and Methods." Calsequestrin was immunoprecipitated from each sample, and scintillation counts were obtained.

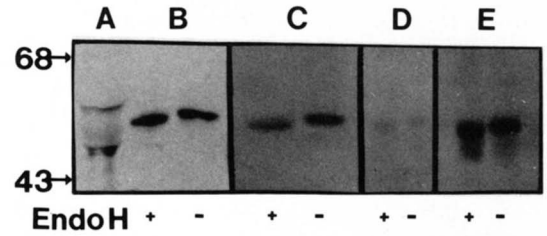


FIG. 6. **Calsequestrin is sensitive to endo H digestion in both CVs and SR and is not secreted.** Chick myotubes were labeled for various times with [^{35}S]methionine, cys and the cells and medium collected and subjected to immunoprecipitation with anti-CSQ followed by SDS-PAGE and fluorography. *A* shows the immunoprecipitate from the medium of cells labeled for 10 h. *B* shows the immunoprecipitates from the cells either treated (+) or untreated (−) with endo H. *C* shows the immunoprecipitated CSQ from CVs isolated from myotubes labeled with [^{35}S]methionine/cysteine for 30 min. *D* shows the immunoprecipitated CSQ from CVs isolated from myotubes labeled for 30 min and then chased for 45 min. *E* shows the immunoprecipitated CSQ from SR isolated from myotubes labeled overnight.

it becomes ricin-agglutinatable or endo H-resistant. We see very similar results with the CV-associated AChE as well, as shown in the following paper (Rotundo *et al.*, 1989). Our interpretation of this data is that one *N*-acetylglucosamine (GlcNAc) residue is added to a terminal mannose of the *N*-linked carbohydrate, 1 h before the other two terminal mannoses are trimmed, thus rendering the molecule endo H-sensitive but able to bind WGA.

We, therefore, determined the ability of CSQ from either SR or CVs to bind to either ConA or WGA columns and to be selectively eluted by either α-methylmannoside or *N*-acetylglucosamine, respectively. Fig. 7 shows the results. Densitometric scanning of the autoradiograms revealed that 62% of the SR-derived CSQ and 58% of the CV-derived CSQ was

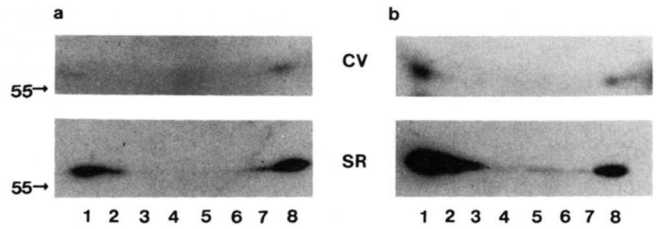


FIG. 7. **Significant amounts of CSQ from CVs and SR, respectively, bind specifically to ConA or WGA columns.** CVs and SR were isolated from 17-day-old chick embryo skeletal muscle as described under "Materials and Methods." Aliquots of each were solubilized in 0.5% Triton X-100, 50 mM Tris (pH 7.4), 0.5% bovine serum albumin and 1 mM Ca^{2+}. Samples were applied to 0.5-ml columns of either ConA or WGA at room temperature, allowed to bind for 30 min, and washed with 4 column volumes of the above buffer. The ConA columns were then washed sequentially with 1 column volume of 300 nM GlcNAc and 1 column volume of 300 mM α-methylmannoside. The WGA columns were treated identically except that the order of addition of GlcNAc and α-methylmannoside was reversed. 20% of each eluted fraction was subjected to SDS-PAGE and immunoblotting with anti-CSQ as described under "Materials and Methods." *a* shows the elution pattern of CV- and SR-derived CSQ from ConA columns. *b* shows the elution pattern of CV- and SR-derived CSQ from WGA columns. In each case, *lane 1* contained the flow through, *lanes 2–5*, and *7* contained washes. In *a*, *lane 6* is the GlcNAc wash and *lane 8* the α-methylmannoside wash. In *b*, *lane 6* is the α-methylmannoside wash and *lane 8* is the GlcNAc wash.

specifically bound to ConA and eluted by α-methylmannoside. 26% of the SR CSQ and 35% of the CV CSQ bound to WGA and was eluted selectively by GlcNAc.

DISCUSSION

The CV-mediated Transport of CSQ to the SR Is Quantitatively Significant—Our data indicate that calsequestrin is a constituent of CVs. In contrast the Ca^{2+}-ATPase which is quantitatively the major SR protein synthesized by embryonic skeletal muscle (reviewed by Martonosi, 1982) is present in undetectable amounts in CVs. These findings argue strongly that the presence of CSQ in CVs is not the result of contamination with SR- or ER-derived membrane vesicles, two potentially significant sources of contamination in view of their high density and tendency to form small right side out vesicles. Based on quantitative calculations, we estimate that there are about 3–4 molecules of CSQ/coated vesicle. We have demonstrated previously that most of the CVs in developing skeletal muscle cells are employed in exocytic processes in that they carry only newly synthesized proteins which have not reached the cell membrane (Benson *et al.*, 1985; Porter-Jordan *et al.*, 1986). The data reported here indicates that all the labeled CSQ in the CVs is endo H-sensitive and chases out of this fraction. Also, none appears to be secreted, thus rendering the possibility that some CSQ is in endocytic CVs untenable. Therefore, we can assume that most of the approximately 2,000 CVs found in a muscle cell equivalent (Robinson, 1982) are involved in exocytosis, and that there are approximately 8,000 molecules of CSQ being transported via CVs to the SR, their presumed destination, at any time. If each CV transit cycle takes 4 min, an estimate based on the kinetics of the delivery of ligands transferred from surface coated pit to endosomes (Anderson *et al.*, 1977), 120,000 newly synthesized CSQ molecules can be transported to the SR/h. Based on our findings that there is 0.5 μg of CSQ/1 mg of protein in 3-day muscle cultures and that there is 1 ng of protein/1 muscle cell equivalent (Stamatos, 1985), we can determine that a muscle cell equivalent contains 2,000,000 CSQ molecules. Since the $t_{1/2}$ of CSQ in cultured muscle cells is 24 h (Zubrycka and MacLennan, 1976), we can easily account for the transport of essentially all newly synthesized CSQ to the SR through a CV-mediated pathway.

In the following paper (Rotundo *et al.*, 1989), we present evidence that the large majority of AChE molecules found in CVs are endo H-sensitive. These data combined with those discussed above are consistent with the view that the major quantitative use of CVs in developing muscle cells is to carry material to the SR. This is not surprising, since the SR represents the largest single membrane compartment in skeletal muscle and its synthesis is maximal at this time (Martonosi, 1982).

The CV-mediated Transport of CSQ to the SR Is Selective—Based on the data shown in Fig. 6, *A* and *B*, it appears that no detectable CSQ becomes endo H-resistant or gets secreted. This is in contrast to the results found with newly synthesized AChE which demonstrate that in both CVs and whole cells, a small but significant fraction becomes endo H-resistant and is subsequently secreted (Rotundo, 1988; Rotundo *et al.*, 1989). Since both proteins are soluble molecules, it would appear that CSQ must bind to a membrane associated constituent, *i.e.* receptor, in order to be selectively segregated from the AChE, which we assume travels the same route as bulk exocytic membrane and secretory transport. The evidence that CSQ is found in only the terminal cisternal elements of the SR (Jorgensen *et al.*, 1983) also argues strongly that it is bound selectively to a membrane associated "receptor" molecule in the lumen of the terminal SR cisternae. Recently, a putative CSQ receptor has been directly visualized in the terminal SR cisternae (Franzini-Armstrong *et al.*, 1987) and also a putative CSQ binding protein from the SR has been identified (Mitchell *et al.*, 1988).

The most parsimonious explanation for these data is that CSQ binds to a receptor molecule located in the early-intermediate Golgi apparatus which is recognized by clathrin and/or associated proteins, *e.g.* the 100-kDa protein (Unanue *et al.*, 1981), and is transported to the SR. CSQ and its receptor remain associated and migrate to the terminal cisternae. There, the receptors are somehow rendered immobile, possibly by association with the T-tubular system. Alternatively CSQ, its receptor and the associated membrane actually stay together after fusion with the Ca^{2+}-ATPase containing SR. In support of this model is the fact that CSQ appears to be anchored to the junctional cisternae (Franzini-Armstrong *et al.*, 1987) and that the Ca^{2+}-ATPase is excluded from the terminal junctional cisternae (Franzini-Armstrong, 1975; Jorgensen *et al.*, 1982).

The Cis-medial Golgi Is the Origin of the CSQ Containing CV—Our data in aggregate demonstrate that all of the CV- and SR-associated CSQ molecules are endo H-sensitive, while approximately 30% of these molecules are specifically bound to a WGA column (Figs. 6 and 7). Since it is very likely that only one terminal GlcNAc can be added to the *N*-linked, endo H-sensitive carbohydrate, and it is also highly probable that CSQ has only one *N*-linked oligosaccharide (Fliegel *et al.*, 1987), it is likely that 30% represents an appreciable underestimate of the true percentage of CSQ molecules which have been terminally *N*-acetylglucosaminated. The reason for this assertion is that the affinity of CSQ for WGA is likely to be much lower than that of glycoproteins which contain several moles of GlcNAc and sialic acid residues.

Therefore, the likely structure of the *N*-linked carbohydrate on CSQ is a -$GlcNAc_2$-$Man_5GlcNAc$, since only terminal GlcNAcs can bind to WGA (Tartakoff and Vassalli, 1983), and the first terminal GlcNAc is added after the removal of 4 terminal mannose residues (Hubbard and Ivatt, 1981). This

structure is still endo H-sensitive. Before further GlcNAcs are added, 2 more terminal mannoses must be removed, rendering the carbohydrate endo H-resistant (reviewed in Kornfeld and Kornfeld, 1985). We hope to confirm this structure using [^{3}H]mannose-labeled cells.

Rothman's group has offered biochemical and cytochemical data indicating that the enzyme which adds the first terminal GlcNAc is in the intermediate or medial Golgi stacks in liver and Chinese hamster ovary cells (Dunphy and Rothman, 1983; Dunphy *et al.*, 1985). This fact would lead to the conclusion that the likely origin of the CSQ containing CV is the intermediate Golgi. However, the unusual nature of the skeletal muscle Golgi apparatus is indicated by its containing only 2–4 stacks (Fambrough and Devreotes, 1978).[2] Skeletal muscle Golgi is also very sluggish in protein transport as indicated by the long residence time of both AChR and AChE in this structure (Fambrough and Devreotes, 1978; Rotundo, 1984) and also by the 1-h lag between terminal GlcNAc addition to AChE and its becoming endo H-resistant (Rotundo, 1984). Therefore, it is uncertain whether the distinction between early and intermediate Golgi holds here. It is, however, safe to conclude that the origin of this CV is before the trans-most stack(s) of the Golgi in which the terminal galactoses are added (Roth and Berger, 1982) since only endo H-sensitive CSQ is found in either CVs or SR or in whole homogenates, and no galactose residues can be added to glycoproteins until the carbohydrate becomes endo H-resistant (reviewed in Kornfeld and Kornfeld, 1985).

Insights into the Formation of the SR—Since we find a relatively large amount of CSQ but no detectable Ca^{2+}-ATPase in isolated CVS, our data support the hypothesis that CSQ reaches the developing SR by a different route than that taken by the Ca^{2+}-ATPase, as first proposed by Jorgensen *et al.* (1977). Since CSQ begins to be synthesized much earlier (at least 10 h) than the Ca^{2+}-ATPase, (reviewed in Martonosi, 1982; MacLennan *et al.*, 1985b) the CSQ containing vesicles, presumably after losing their clathrin coats, may serve as the nucleating sites for the addition of Ca^{2+}-ATPase containing membrane.

Relevance of the CV-mediated CSQ Transport System to Other Cell Types—Is the finding of a quantitatively major transport pathway in developing skeletal myotubes between the Golgi and SR, which is mediated by CVs carrying endo H-sensitive glycoproteins, of any significance with respect to non-muscle cells? Rothman and Fine (1980) and, subsequently, Kinnon and Owen (1983) have reported the existence of an early wave of CVs carrying newly synthesized, endo H-sensitive glycoproteins in Chinese hamster ovary cells and plasmacytoma cells, respectively. Potentially these could represent the same pathway as that described here.

Recently, the existence of an SR-like membrane system which releases Ca^{2+} in response to inositol trisphosphate, an intracellular second messenger produced by many hormone-receptor system (reviewed in Berridge, 1984) has been demonstrated in the cortex of sea urchin eggs (Oberdorf *et al.*, 1986). A protein very similar to CSQ in this organelle has been characterized (Oberdorf, *et al.*, 1988). Also a protein similar to CSQ has been shown to be present in liver endoplasmic reticulum (Damiani *et al.*, 1988) and we have recently found evidence of a CSQ-like protein in bovine brain synaptosomes as well (Benson and Fine, 1987). Also very recently, the existence of a membranous, calsequestrin and inositol trisphosphate receptor containing organelle in several vertebrate non-muscle cells has been reported (Volpe *et al.*, 1987). We are presently investigating the possible role of CVs in the formation of this organelle.

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[2] R. Benson, unpublished observation.

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