Vesicle budding from endoplasmic reticulum is involved in calsequestrin routing to sarcoplasmic reticulum of skeletal muscles

Alessandra NORI, Elena BORTOLOSO, Federica FRASSON, Giorgia VALLE and Pompeo VOLPE[1]

Dipartimento di Scienze Biomediche Sperimentali dell'Università di Padova, viale G. Colombo 3, 35121 Padova, Italy

CS (calsequestrin) is an acidic glycoprotein of the SR (sarcoplasmic reticulum) lumen and plays a crucial role in the storage of Ca^{2+} and in excitation–contraction coupling of skeletal muscles. CS is synthesized in the ER (endoplasmic reticulum) and is targeted to the TC (terminal cisternae) of SR via mechanisms still largely unknown, but probably involving vesicle transport through the Golgi complex. In the present study, two mutant forms of Sar1 and ARF1 (ADP-ribosylation factor 1) were used to disrupt cargo exit from ER-exit sites and intra-Golgi trafficking in skeletal-muscle fibres respectively. Co-expression of Sar1-H79G ($His^{79} \rightarrow Gly$) and recombinant, epitope-tagged CS, CSHA1 (where HA1 stands for nine-amino-acid epitope of the viral haemagglutinin 1), barred segregation of CSHA1 to TC. On the other hand, expression of ARF1-N126I altered the subcellular localization of GM130, a *cis*-medial Golgi protein in skeletal-muscle fibres and myotubes, without interfering with CSHA1 targeting to either TC or developing SR. Thus active budding from ER-exit sites appears to be involved in CS targeting and routing, but these processes are insensitive to modification of intracellular vesicle trafficking and Golgi complex disruption caused by the mutant ARF1-N126I. It also appears that CS routing from ER to SR does not involve classical secretory pathways through ER–Golgi intermediate compartments, *cis*-medial Golgi and *trans*-Golgi network.

Key words: calsequestrin, sarcoplasmic reticulum, skeletal muscle, targeting.

INTRODUCTION

SR (sarcoplasmic reticulum) of skeletal muscles, a network of tubules (longitudinal SR) and TC (terminal cisternae) devoted to intracellular Ca^{2+} homoeostasis [1,2], comprises two continuous membrane domains, the non-junctional SR enriched in Ca^{2+}-pump and the junctional SR juxtaposed to the transverse tubules and enriched in Ca^{2+} release channel (also known as ryanodine receptor, RYR). CS (calsequestrin), an acidic, low-affinity, high-capacity (40–50 mol/mol) Ca^{2+}-binding glycoprotein [3], segregates at the internal face of the junctional SR in the TC lumen [4,5] and plays a crucial role in Ca^{2+} release [1]. In fact, binding of Ca^{2+} to CS (see [6] and references therein) and changes of the phosphorylation state of CS [7,8] influence, directly and indirectly, the activity of the RYR-Ca^{2+} release channel.

The SR is a subcompartment of the ER (endoplasmic reticulum) as indicated by the co-existence of ER general markers, i.e. BiP (immunoglobulin heavy-chain binding protein), calnexin and PDI (protein disulphide isomerase), with specific molecular components of Ca^{2+} stores, e.g. CS, Ca^{2+} pump and RYR [2]. The molecular differentiation of SR occurs from an ER-derived reticular network, includes at an early stage the concentration of CS within membrane-bound structures and progressively evolves into the establishment of triads [9,10].

Since the structure of the N-linked oligosaccharide of mammalian skeletal muscle CS is reported to be $GlcNAc_2$-Man_3 and $GlcNAc_2$-Man_1 [1,3,4,11], and to be endoglycosidase-H-insensitive [12], processing through the GC (Golgi complex) appears plausible. A stage through the GC has been described for CS in developing skeletal myotubes of the chicken [13]. In contrast, Gatti et al. [14] observed recombinant CS in ER subdomains of L6 myoblasts, found CS to be completely endoglycosidase-H-sensitive, and proposed that CS never traffics through the GC. CSHA1ΔGly, a mutant in which the unique N-glycosylation site of CS (Asn^{316}) was changed to isoleucine, and, thus, not able to undergo the initial ER glycosylation, has been shown to segregate to TC *in vivo* [15]. Whether and how CS exits from ER and cycles through the GC are, at present, still unresolved questions.

With this aim, we perturbed the intracellular traffic between ER/SR and the GC, both in skeletal-muscle fibres and cultured myotubes, with a dominant-negative mutant of the small G-protein ARF1 (ARF1N126I, where ARF1 stands for ADP-ribosylation factor 1) and a constitutively active form of the Sar1-GTPase (Sar1-H79G) and observed the effects on CS targeting. ARF1 is a component of the ERGIC (ER–Golgi intermediate compartment) and intra-Golgi COPI (coat protein complex I) trafficking vesicles and is required to form clathrin-coated vesicles containing AP1 (adaptor protein 1) or AP3 adaptor complexes at the TGN (*trans*-Golgi network). ARF1-N126I is defective in GTP binding and, thus, inhibits ER export [16]. ARF1 and COPI mediate and regulate the budding of vesicles carrying specific cargo proteins on Golgi membranes [17]. When a COPI assembly is blocked with either ARF1 mutants or BFA (Brefeldin A), retrograde membrane traffic is deregulated and Golgi proteins may enter a constitutive, non-selective tubular pathway back to ER [17,18]. So far, ARF1 and its mutants have been used only in cell models [16,19,20]. ARF1-N126I affects both the classical exocytotic pathway for glycoproteins, either secreted or sorted to the plasma membrane in mononucleated cells, and the apical sorting in polarized epithelial cells. Sar1-GTPase is a component of the COPII, which is assembled on specific sites of ER membranes

Abbreviations used: AP, adaptor protein; ARF1, ADP-ribosylation factor 1; BFA, Brefeldin A; BiP, immunoglobulin heavy-chain binding protein; COPI, coat protein complex I; CS, calsequestrin; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment; GC, Golgi complex; GFP, green fluorescent protein; HA1, nine-amino-acid epitope of the viral haemagglutinin 1; PDI, protein disulphide isomerase; RYR, ryanodine receptor; SR, sarcoplasmic reticulum; TC, terminal cisternae; TGN, *trans*-Golgi network; VSV-G, vesicular-stomatitis-virus glycoprotein.

[1] To whom correspondence should be addressed (e-mail pompeo.volpe@unipd.it).

(ER-exit sites) to form COPII vesicles. Sar1 is activated by Sec12p guanine nucleotide exchange factor to Sar1-GTP, which transiently associates with specific cargos and promotes COPII coat polymerization and cargo selection. Conversion of Sar1-GTP into Sar1-GDP allows maturation of the coat and promotes vesicle budding. The GTP-bound mutant Sar1-H79G induces accumulation of COPII vesicles [21] and blocks efficient export of specific cargoes, e.g. VSV-G (vesicular-stomatitis-virus glycoprotein), from the ER in cell cultures [22].

In recent years, classical exocytotic pathways to the plasma membrane/transverse tubule and trafficking between ER–GC have been elegantly dissected with probes derived from enveloped viral glycoproteins in differentiating myoblasts and isolated muscle fibres (see [23] and references therein). In contrast, routing to and/or from SR, both during differentiation and in fully differentiated muscle fibres, has not been thoroughly investigated. In the present study, we investigated CSHA1 (where HA1 stands for nine-amino-acid epitope of the viral haemagglutinin 1) targeting on inhibition of ER export and Golgi disruption to address key issues pertaining to CS routing to TC *in vivo*.

EXPERIMENTAL

Generation of cDNAs

The cDNA corresponding to CSHA1 was developed as described previously [24]. ARF1-c-*myc* and ARF1-N126I were obtained from D. H. A. Jones (Department of Physiology, University College, London, U.K.) and subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen, san Giuliano Milanese, Italy). Orientation and correct sequence of ARF1-c-*myc* and ARF1-N126I were checked by restriction assays and sequencing. ER-GFP (green fluorescent protein) was from Invitrogen. cDNA coding for Sar1-H79G-HA1 was obtained from Dr J. Lippincott-Schwartz (Cell Biology and Metabolism Branch, National Institutes of Health, Bethesda, MA, U.S.A.).

Tissue sources and materials

Soleus, a representative slow-twitch skeletal muscle, was used for *in vivo* transfection. After the animal was killed, soleus muscles were quickly removed and immediately frozen in liquid nitrogen for biochemical analysis or fixed in paraformaldehyde for immunofluorescence.

DMEM (Dulbecco's modified Eagle's medium) and its complement were purchased from Sigma. DNA modification and restriction enzymes were from Promega (Milano, Italy). All other chemicals were from Sigma.

Cell cultures

C_2C_{12} myoblasts [25], subcloned from the C_2 mouse myoblast cell line, were grown in DMEM (high glucose) supplemented with 10 % (v/v) foetal calf serum (proliferation medium). Cells were seeded on 0.02 % gelatin-coated round coverslips placed on 24-well-culture plates. When approaching confluence, C_2C_{12} myoblasts were induced to differentiate and fuse into multinucleated myotubes by changing the proliferation medium to DMEM with 2 % (v/v) horse serum (differentiation medium). BFA treatment was performed as described in [26].

Generation of transgenic skeletal-muscle fibres and transient transfectants of C_2C_{12} myotubes

Male adult Wistar rats (approx. 250 g of body weight) were anaesthetized with ketamine (1.5 mg/100 g of body weight). Soleus muscles were exposed and injected with 0.06 ml of saline solution containing 50 μg of cDNAs for single transfection and 100 μg for co-transfection. Electroporation was performed with a BTX ECM 830 square-wave pulse generator according to the method of Mathiesen [27], as described previously [28]. Treated rats were killed 2, 6, 7 or 14 days after surgery.

Myotubes seeded on gelatin-coated coverslips were transfected with cDNAs (either 20 or 40 μg/well for co-transfection) by electroporation. The cells, 24 h after transfection, were fixed for immunofluorescence.

Immunofluorescence

Myotubes were fixed in 4 % (w/v) 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 % (v/v) swine serum (buffer A) for 30 min. Both transversal (9 μm) and longitudinal (6 μm) sections were obtained from soleus skeletal muscles, as described in [24]. Incubation with monoclonal (Roche, Monza, Italy) and polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) anti-HA1 antibodies, monoclonal anti-c-Myc antibodies (Roche), monoclonal anti-GM130 antibodies (BD Transduction Laboratories, San Jose, CA, U.S.A.), polyclonal anti-p58 antibodies [29] and polyclonal anti-calreticulin antibodies (Affinity Bioreagents, Vinci Firenze, Italy) was performed at room temperature for 90 min in buffer A. After 60 min washing, either cells or muscle sections were incubated for 30 min with rhodamine isothiocyanate (Sigma–Aldrich, Milano, Italy), Cy2-conjugated anti-mouse (Chemicon, Milano, Italy) or fluorescein-conjugated anti-rabbit (Dako, Milano, Italy) antibodies. Images were obtained with a Leica DMRB microscope.

RESULTS

Expression of ARF1-c-*myc* and Sar1-H79G-HA1 in transgenic skeletal-muscle fibres and in C_2C_{12} myotubes

The expression and subcellular localization of both wild-type ARF1-c-*myc* and Sar-1H79G-HA1 were studied in transgenic skeletal-muscle fibres of the adult rat, 2 days after *in vivo* transfection and electroporation. Expression and subcellular localization of wild-type ARF1-c-*myc* were also studied in cultured C_2C_{12} myotubes, transfected by electroporation [30].

On transfection with either ARF1-c-*myc* or Sar1-H79G cDNAs, positive skeletal-muscle fibres were observed 2 days after electroporation in transverse muscle sections labelled with anti-c-Myc and anti-HA1 antibodies respectively (results not shown): in both cases, the signal is intense at the periphery, where 75 % of all GC elements are located in type 1 slow-twitch fibres, although different levels of expression are recorded among the fibres [31].

To verify the specificity of ARF1-c-*myc* distribution and identify labelled subcellular compartments, we studied the localization of recombinant ARF1-c-*myc* in relation to that of endogenous p58, a known ERGIC marker [32], in both C_2C_{12} myotubes and transgenic skeletal-muscle fibres (Figure 1). In C_2C_{12} myotubes, ARF1-c-*myc* had a widespread distribution (Figure 1A); in some cases, perinuclear accumulation could be observed. This labelling is consistent with the subcellular distribution of ARF1: GDP-bound ARF is soluble in the cytosol, whereas the active GTP-bound form is tightly associated with endomembranes. ARF1-c-*myc* distribution was partially similar to that of endogenous p58 (Figure 1B) observed only in the ERGIC, and consisted of both perinuclear structures and disperse cytoplasmic dots in myotubes (cf. [33]). c-*myc*-negative, p58-positive structures indicate that ARF-c-*myc*, even if overexpressed, is specifically and not homogeneously associated with a subset of ERGIC components;

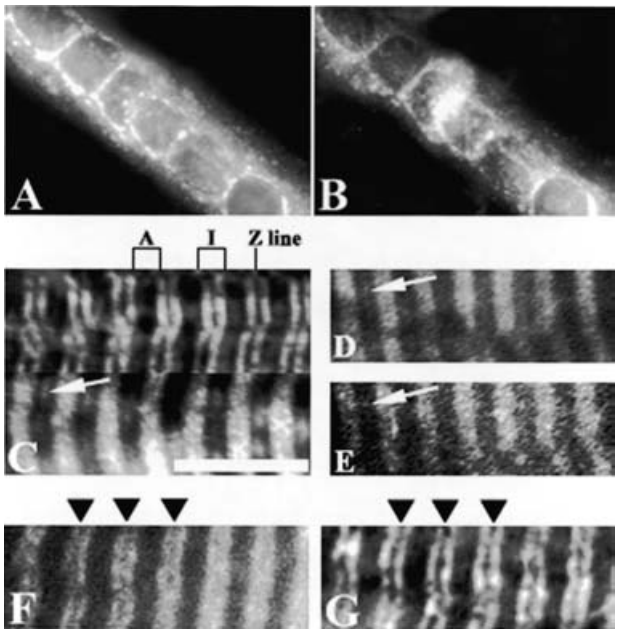

Figure 1 Localization of ARF1-c-*myc* and Sar1-H79G-HA1 in skeletal-muscle fibres and C_2C_{12} myotubes

Double immunofluorescence of C_2C_{12} myotubes (**A**, **B**) and longitudinal sections of skeletal-muscle fibres (**C**–**G**) after transfection with ARF1-c-*myc* (**A**–**E**) and Sar1 H79G-HA1 (**F**, **G**) cDNAs. Fixed myotubes were sequentially decorated with monoclonal anti-c-*myc* (**A**) and polyclonal anti-p58 (**B**) antibodies. Longitudinal sections of transgenic skeletal-muscle fibres were stained with anti-c-Myc antibodies (lower part of **C** and **D**) and anti-p58 antibodies (**E**). The soluble form of ARF1-c-*myc* was also detected because no permeabilization was performed before fixation. Upper part of (**C**) represents the relative contrast-phase image. Sar1-H79G-HA1-transfected fibres were stained with monoclonal anti-HA1 antibodies (**G**); (**F**) represents the contrast-phase image of the same area. Arrowheads point to Z lines and arrows point to staining in the middle of the A band. Scale bars: (**A**, **B**), 37 μm and (**C**–**G**), 8 μm.

c-*myc*-positive, p58-negative structures indicate that compartments other than ERGIC are labelled. Analysis of longitudinal muscle sections shows the localization of recombinant ARF1-c-*myc* (Figure 1, lower part of C) in relation to the sarcomeric structure (Figure 1, upper part of C): ARF1-c-*myc* displayed a cross-striated staining distributed homogeneously at the level of the I band along with a regular thin line in the middle of the A band. Moreover, double immunofluorescence analysis shows that ARF1-c-*myc* (Figure 1D) and p58 (Figure 1E) were present in the same areas and partially co-localized both in the I band and in the middle of the A band. p58-positive structures at the A band were also identified in normal, non-transfected muscles and the signal specificity was confirmed in control sections processed with either one or both of the secondary antibodies (results not shown). In longitudinal muscle sections, the localization of recombinant Sar1-H79G is shown (Figure 1F) in relation to the sarcomeric structure (Figure 1G). The cross-striated staining extended over the I band, decreased with respect to the Z line, whereas the thin line in the middle of the A band was occasionally detectable. The sarcomeric organization was preserved in positive fibres. These results indicate that (i) localization of Sar1-H79G is compatible with that of ER/SR markers in muscle fibres [34]; and (ii) the pool of ARF1-c-*myc* is *bona fide* associated with ERGIC.

Expression of ARF1-N126I in transgenic skeletal-muscle fibres alters GM130 distribution but does not modify CS targeting to SR

GM130 is a suitable indicator of ER–GC traffic in muscle systems because (i) it is a component of the *cis*-medial Golgi and its distribution is fibre-type-dependent in muscle [31]; (ii) in single muscle fibres, it has been used to monitor *cis*-medial Golgi modifications caused by nerve activity [35]; and (iii) in myotubes, GM130 compartments have also been identified in the 'near proximity' of ER exit sites [26].

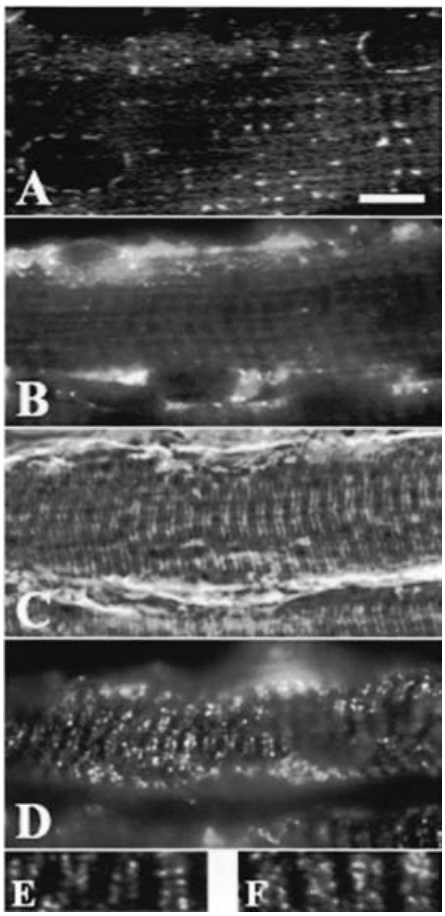

Figure 2 Transgenic skeletal-muscle fibres obtained 2 days after co-transfection with cDNAs coding for ARF1-N126I and CSHA1: disruption of GM130 labelling pattern is associated with normal subcellular distribution of CSHA1

All muscle sections were obtained 2 days after electroporation. Longitudinal sections were sequentially incubated with monoclonal anti-GM130 antibodies (**A**, **B**) and polyclonal anti-HA1 antibodies (**D**–**F**). (**A**) GM130 distribution in electroporated but CSHA1-negative fibre. (**B**–**D**) Same fibre showing an altered distribution of GM130 (**B**), normal sarcomeric structure (contrast-phase image of **C**) and normal CSHA1 distribution (**D**). (**E**, **F**) $\times$ 2.5 magnification of control and co-transfected fibres respectively, showing the typical CS pattern, i.e. parallel rows of bright spots, each resulting from clusters of TC filled with CS. Five co-transfection experiments were performed. Scale bar, 3.2 μm.

In this experimental series, soleus skeletal muscles were co-transfected by electroporation with ARF1-N126I and either ER-GFP (results not shown) or CSHA1. Figure 2 shows the labelling pattern of anti-GM130 and anti-HA1 antibodies in transgenic skeletal-muscle fibres obtained 2 days after co-transfection. Figure 2(A) shows electroporated fibres negative for both CSHA1 and ARF1-N126I: GM130 was in discrete structures, either convolute, perinuclear ribbons or scattered in orderly fashion throughout the cytoplasm. Figures 2(B)–2(D) depict representative co-transfected fibres expressing CSHA1 and ARF1-N126I: GM130 distribution was altered (Figure 2B), i.e. all discrete structures were disarrayed and replaced by perinuclear clouds, whereas sarcomeric structures were well preserved (Figure 2C) and a regular

banding pattern was revealed by anti-HA1 antibodies (Figure 2D). At higher magnification, the typical CSHA1 pattern, i.e. parallel rows of bright spots each resulting from clusters of TC filled with CS [15], was observed both in control fibres transfected with CSHA1 (Figure 2E) and in co-transfected fibres (Figure 2F). Overall, it appears that expression of ARF1-N126I in transgenic skeletal-muscle fibres, up to 2 days, specifically determines disassembly of the GC and of unidirectional ER–GC trafficking, as indicated by disruption of GM130 labelling, without interfering with CS targeting to SR.

Expression of ARF1-N126I in C_2C_{12} myotubes: effects on GM130 distribution and CSHA1 targeting

The main evidence that CS targeting is supported by vesicle-mediated transport derives from isolation and purification of CS-rich clathrin-coated vesicles in chicken skeletal-muscle myotubes [13]. To confirm the activity of the dominant-negative probe in C_2C_{12} myotubes, we compared the effects of ARF1-N126I with those of BFA on the subcellular distribution of GM130.

The GM130 labelling pattern of ER-GFP-transfected myotubes (Figure 3C) was comparable with control myotubes (Figure 3A), indicating that electroporation itself does not affect GC organization (see also [30]). The effect of ARF1-N126I on GM130 distribution is shown in Figure 3(D). Co-transfected, ER-GFP-positive myotubes, identified by direct fluorescence, were devoid of discrete, perinuclear structures but displayed a homogeneous cytoplasmic fluorescence, and a punctuate pattern that resembled the BFA effect reported in Figure 3(B) (see also [26,36]).

The distribution of endogenous CS and/or CSHA1 was compared in control, CSHA1-transfected and ARF1-N126I/CSHA1-co-transfected myotubes. A representative distribution pattern of endogenous CS in control, non-transfected and non-electroporated large myotubes is shown in Figure 4(A), i.e. several discrete, peripheral CS clusters were observed. Comparison of CS labelling in control myotubes with CSHA1 labelling in either singly transfected (Figure 4B) or co-transfected (Figure 4C) myotubes shows the presence of discrete, regularly spaced peripheral spots. Such structures resembled those observed with antibodies to CS in rat primary myotubes and suggest the occurrence of a muscle-specific developing compartment [32]. Two ER-specific markers, calreticulin (Figure 4D) and ER-targeted GFP (Figure 4E), displayed a distribution pattern clearly different from that of CSHA1. 'Nascent SR' was described in L6 cell lines as an endomembrane subcompartment that develops during myogenesis, and expresses CS with a distribution distinct from that of calreticulin [37] and other ER markers [38,39]. Thus, even if SR never attains complete differentiation in cultured myotubes, we can distinguish a differentiating intracellular compartment in C_2C_{12} myotubes that could represent developing SR.

Comparison of Figure 4(C) with Figures 4(A) and 4(B) indicates that CSHA1 distribution in ARF1-N126I-expressing myotubes resembled that of endogenous CS and was the same in the absence or presence of ARF1-N126I. Overlay between GM130 and HA1 signals in GC-disrupted myotubes is shown in Figure 4(C′): the overall lack of co-localization indicates that block of GC–ER retrograde transport did not induce accumulation of CSHA1 to recycling compartments. Thus a tentative conclusion could be that in differentiated myotubes, despite disarray of GC and blocking of unidirectional ER–GC trafficking, CSHA1 is normally sorted out. In conclusion, both in C_2C_{12} myotubes and transgenic skeletal-muscle fibres, expression of ARF1-N126I affected GM130 distribution and did not interfere with CSHA1 targeting.

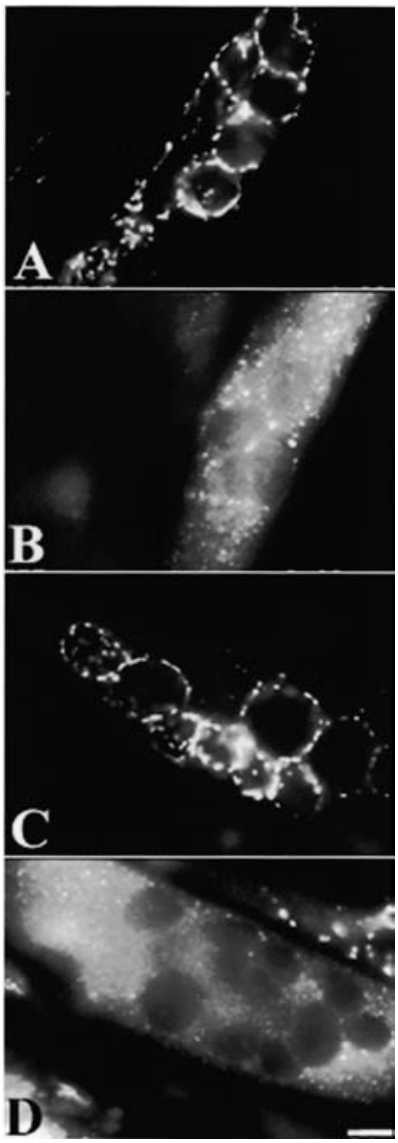

Figure 3 Effect of BFA and ARF1-N126I on subcellular distribution of GM130 in C_2C_{12} myotubes

Control myotubes (**A**), myotubes incubated for 1 h with BFA (**B**), myotubes transfected with cDNA coding for ER-GFP (**C**), myotubes co-transfected with cDNAs coding for ARF1-N126I and ER-GFP (**D**). (**C**, **D**) Myotubes were GFP-positive, as indicated by direct fluorescent detection. Fixed myotubes were decorated with anti-GM130 antibodies. Experiments were performed on seven different myotube preparations and results represent the typical immunofluorescence pattern. Scale bar, 10 μm.

Expression of Sar1-H79G affects sorting of CSHA1 to TC in transgenic skeletal-muscle fibres

To assess whether exit from ER is a prerequisite for CS targeting to SR, expression and localization of CSHA1 were studied in transgenic skeletal-muscle fibres, 2 days after co-transfection with Sar1-H79G-HA1. Under the prevailing experimental conditions, only the monoclonal anti-HA1 antibody recognized Sar1-H79G-HA1 (see Figure 1); thus, in co-transfected muscles, polyclonal anti-HA1 antibodies exclusively recognized CSHA1. Figure 5 is representative of the fluorescent pattern obtained with anti-HA1 antibodies in longitudinal sections of transfected (CSHA1; Figures 5A and 5A′) and co-transfected (Sar1-H79G plus CSHA1; Figures 5B and 5B′) fibres. The characteristic regular banded

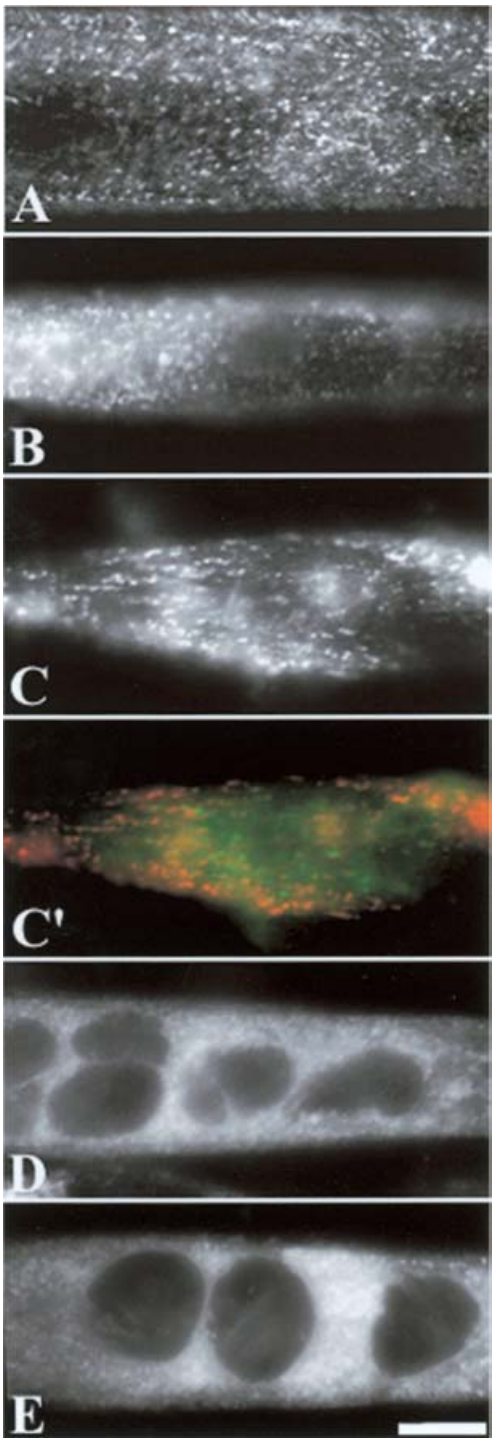

Figure 4 Effect of ARF1-N126I on CSHA1 distribution in C_2C_{12} myotubes co-transfected with cDNAs coding for ARF1-N126I and CSHA1

Control, non-transfected myotubes (**A**) were fixed and decorated with monoclonal anti-CS antibodies. Myotubes, either transfected with CSHA1 (**B**) or co-transfected with ARF1-N126I and CSHA1 (**C**) were fixed and decorated with anti-HA1 antibodies. (**C′**) The merge image obtained with anti-GM130 antibodies (green) and anti-HA1 antibodies (red). (**D**, **E**) ER-GFP transfected myotubes were either decorated with polyclonal anti-calreticulin antibodies or directly observed respectively. Experiments were performed on six different myotube preparations and results represent the typical immunofluorescence pattern. Scale bar, 10 μm.

pattern (cf. Figure 2E) was lost on expression of Sar1-H79G and was replaced by a faint signal homogeneously distributed all over the I band with sporadic condensation at the A–I boundaries. Moreover, concentration of the fluorescent signal was occasionally observed at the Z line (results not shown). The distribution of recombinant CS in the presence of Sar1-H79G is compatible with that of general ER (BiP or PDI) and rough ER (RibII) markers [2,34]. These results clearly indicate that Sar1-H79G interferes with CSHA1 targeting to TC.

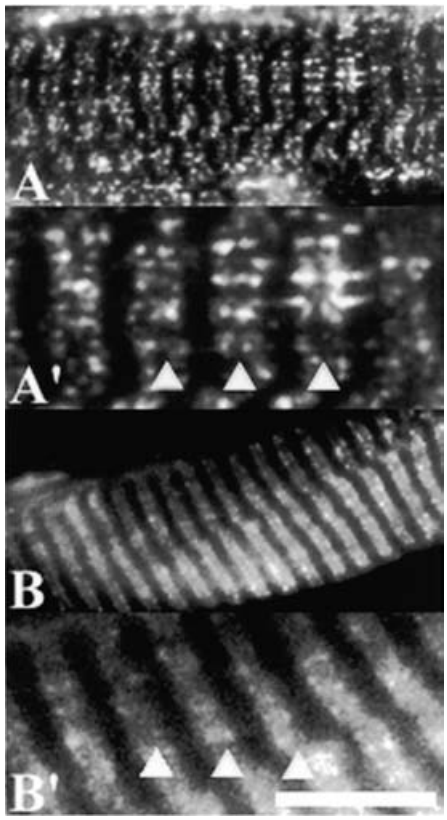

Figure 5 CSHA1 distribution in skeletal-muscle fibres obtained 2 days after co-transfection with cDNAs coding for Sar1-H79G and CSHA1: effect of Sar1-H79G on CSHA1 targeting

Longitudinal sections obtained from CSHA1-transfected (**A**, **A′**) and CSHA1-Sar1 H79G-co-transfected (**B**, **B′**) soleus muscles were sequentially decorated with polyclonal anti-HA1 antibodies and TRITC (tetramethylrhodamine β-isothiocyanate)-conjugated anti-rabbit antibodies. The regular banded pattern of the HA1 signal displayed in (**A′**) and representative of normal CS clustering to TC (see also legend to Figure 2) is lost in Sar1-H79G-co-transfected fibres (**B′**). Arrowheads point to consecutive Z lines. Two co-transfection experiments were performed. Scale bars: (**A**, **B**), 3.2 μm and (**A′**, **B′**), 8 μm.

DISCUSSION

The present study reports the effects of (i) ARF1-N126I on GC of soleus skeletal-muscle fibres of the adult rat and of cultured myotubes, and (ii) Sar1-H79G on CSHA1 targeting to TC in skeletal-muscle fibres. We show that CS routing to SR is sensitive to Sar1-H79G, whereas ARF1-N126I-sensitive pathways appear not to be involved. This was achieved by direct electroporation of cDNAs referable to the GTP-bound-mutant Sar1-H79G and the nucleotide-free dominant-negative mutant ARF1-N126I. For the latter mutant, effective GC disruption has been demonstrated by monitoring the intracellular distribution of GM130; disarray of GM130 labelling, taken as direct evidence of blockade of ARF1-N126I-sensitive ER–GC pathways, took place in parallel with proper targeting of CS. On the other hand, *in vivo* evidence has been obtained indicating that vesicular pathways downstream

of ER and active ER-exit sites are involved in CS targeting to TC.

Intracellular localization of ARF1 and Sar1-H79G

We have shown that wild-type ARF1 distributes to intracellular compartments of both skeletal-muscle fibres and myotubes and partially co-localizes with the ERGIC marker p58. The complete co-localization in perinuclear structures of C_2C_{12} myotubes indicates that ARF1-c-*myc* is associated with Golgi perinuclear membranes where other known GC markers, such as α-mannosidase II, giantin, TGN38, GM130 and GM160, are concentrated [33,40]. Disperse cytoplasmic dots, on the other hand, show partial co-localization with p58 in agreement with the finding that ARF1 in mononucleated cells is a component of the GC [40], ERGIC [41] and TGN [42].

In transgenic skeletal-muscle fibres, both ARF1 and Sar1-H79G are expressed in two sarcomeric domains: the I band and the centre of the A band. The partial co-localization of ARF1-c-*myc* with p58 indicates that a selective but not homogeneous association with distinct compartments occurs *in vivo*. The assumption that localization of ARF1-c-*myc* reflects that of endogenous ARF1 at the I band is supported by the finding that active Golgi trafficking of VSV-G involves at least COPI vesicles and occurs near the fenestrated longitudinal SR [43]. Moreover, localization of the mutant Sar1 at the I band with a labelling signal similar to that of general ER markers [2,34], confirms that SR and ER are present in the same sarcomeric area. On the other hand, both proteins display a thin labelling at the M line, indicating that other compartments could be involved in vesicle trafficking between ER/SR and GC; at the M line level of skeletal-muscle fibres, two KDEL-soluble proteins (BiP and PDI) and a membrane chaperone (calnexin) are expressed [2], confirming that recycling via KDEL receptors should take place in this region.

Localization of the Golgi matrix protein GM130 is affected by expression of ARF1-N126I in transgenic skeletal-muscle fibres and in myotubes

We have shown that expression of ARF1-N126I disrupts GM130 localization in both transgenic skeletal-muscle fibres and myotubes. These findings suggest that ARF1 is involved in vesicle trafficking in muscle fibres *in vivo* and that GC markers redistribute to other intracellular compartments on COPI and/or AP1–AP3 disassembling from the GC.

In ARF1-N126I-transfected myotubes (Figure 3), GM130 redistributes into discrete structures distinct from the whole ER. Such structures are referable to either GC remnants scattered throughout the cytoplasm or compartments located between ER-exit sites and GC remnants [17]. Although it cannot be ruled out that GM130 redistributed to ER subcompartments, as described for giantin in BFA-treated myotubes, our results with ARF1-N126I show that GM130 localization is not comparable with that of GC enzymes, such as α-mannosidase II homogeneously redistributed to reticular ER ([39]; E. Bortoloso and A. Nori, unpublished work).

In skeletal-muscle fibres, ER-exit sites have been identified, using the COPII marker Sec31p [35], as discrete ring-like, perinuclear structures and disperse intermyofibrillar structures aligned with microtubules. Analysis of retrograde trafficking of VSV-G in skeletal-muscle fibres indicated that GM130 and VSV-G appear to concentrate in microtubule-associated structures, after BFA treatment [44]. In ARF1-N126I-transfected muscle fibres (Figure 2), GM130 is enriched in disorganized structures, morphologically different from ER-exit sites, and located in proximity of nuclei where microtubules and microtubule-organizing-centre components concentrate after denervation [31]. Our results also suggest that members of the ARF1 and Sar1 regulator protein families (guanine nucleotide exchange factor and GTPase-activating protein) are constitutively expressed during differentiation and in skeletal-muscle fibres.

Evidence that CS routing depends on active exiting from ER and escapes the block of ARF1-N126I in transgenic skeletal-muscle fibres

The mutant Sar1-H79G affects targeting of VSV-G, i.e. VSV-G is concentrated in the ER [22]; moreover, in isolated skeletal-muscle fibres, efficiently exported proteins concentrate at the I band, preferentially in the proximity of the Z line, if blocked in the ER by BFA [34]. On expression of Sar1-H79G, the subcellular localization of recombinant CSHA1 resembles that of ER markers (Figure 5; see also [2,34]). Thus a plausible interpretation of our results is that correct CS targeting needs active exiting from ER possibly via budding of COPII vesicles. This finding reinforces the notion that CS sorting involves vesicle-mediated transport and is in contrast with the hypothesis that CS directly diffuses from ER to SR. An alternative interpretation could be that incorrect localization of recombinant CS is caused by GC disorganization evoked by Sar1-H79G expression. The results obtained with ARF1-N126I seem to rule out this possibility. In fact, in transgenic skeletal-muscle fibres, CSHA1 is correctly targeted to TC (Figure 2); in myotubes, despite GC disruption, CS and CSHA1 do not concentrate in either ERGIC or modified GM130 compartments, but are sorted out to developing SR (Figure 4). Our findings clearly indicate that *cis*-medial Golgi structures expressing GM130 are not involved in CSHA1 routing and targeting. Since ARF1-N126I also affects sorting of proteins trafficking from ERGIC to TGN, we infer that even these sorting compartments are not involved in CSHA1 routing. Overall, GC disarray evoked by ARF1-N126I does not affect CS targeting.

The existence of muscle-specific routes to SR, originating either from ERGIC or directly from ER, has been proposed to explain (i) the heterogeneity of VSV-G glycosylation, both in differentiated myotubes and isolated fibres [44], and (ii) how several glycoproteins acquire protection from Golgi enzymes in BFA-treated L6 myotubes [45]. Taking our results together, it would appear that CS on exiting from ER travels to SR without reaching *cis*-medial Golgi, as it was described for VSV-G, a bulk flow marker. Moreover, present evidence of *cis*-Golgi bypass for CS targeting strengthens our previous results showing that CSHA1ΔGly, a mutant lacking the unique glycosylation site, was correctly sorted to TC in transgenic skeletal-muscle fibres [15].

Within the limits of the present results, it can be stated that vesicle-mediated transport is involved in CS targeting. Clathrin-coated vesicles containing AP1 and AP3 adaptors, in particular the muscle-specific clathrin homologue CHC22 [46], should be ruled out because such a pathway is ARF1-N126I-sensitive. Previous observations, i.e. CS is sorted via clathrin-coated vesicles in chicken skeletal-muscle myotubes [13], can be reconciled with present findings assuming either the existence of species specificity or that other isoforms of clathrin coat and of clathrin–adaptor complexes are involved. On the other hand, COPII coat components are expressed in muscle tissues [47,48] and our results indicate that they are involved in CS trafficking. It has been confirmed recently [11] that canine skeletal-muscle CS also displays mannose trimming usually ascribed to GC enzymes. The discrepancy with our own evidence might be only apparent based on two distinct lines of reasoning: (i) *in vivo* studies on multinucleated myotubes have recently shown the constitutive

recycling of recombinant mannosidase II-GFP, a classical Golgi marker, indicating the occurrence of peculiar mechanisms for handling Golgi enzymes in muscle [26], i.e. mannose trimming might take place in ER/SR; and (ii) in non-muscle cells, recombinant cardiac CS is retained in the ER and mannose trimming by post-ER mannosidases is ruled out [11], i.e. specific pathways for processing CS glycan are lacking and direct ER–GC transfer is barred. The intriguing possibility would be that transit through SR and interaction with a specific SR receptor are necessary prerequisites for mannose trimming. Thus CS might shuttle to GC only after reaching either developing SR in myotubes or TC in muscle fibres via specific routes characterized by new general as well as muscle-specific, molecular components and regulators [46,49–53].

This work was supported by Telethon, Italy (grant no. 1274) and by funds from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (2001–2003, Programma di ricerca di rilevante interesse nazionale on 'Biopatologia della fibra muscolare scheletrica'). We thank Professor S. Cockcroft and Dr D. H. A. Jones (University College, London, U.K.) for providing cDNAs referable to ARF1 and ARF1-N126I, Dr J. Lippincott-Schwartz and Dr J. Saraste (University of Bergen, Bergen, Norway) for the gifts of Sar1-H79G cDNA and anti-p58 antibodies respectively.

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Received 4 December 2003; accepted 16 January 2004
Published as BJ Immediate Publication 16 January 2004, DOI 10.1042/BJ20031875