Transport pathway, maturation, and targeting of the vesicular stomatitis virus glycoprotein in skeletal muscle fibers

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SUMMARY

We have infected isolated skeletal muscle fibers with the vesicular stomatitis virus or the mutant tsO45, whose glycoprotein is blocked in the endoplasmic reticulum at 39°C. Immunofluorescence analysis for the viral glycoprotein indicated that the fibers were infected over their entire length at a virus dose of 10⁹/ml. When we infected the myofibers with the tsO45 mutant at 39°C, the viral glycoprotein appeared to be localised to the terminal cisternae of the sarcoplasmic reticulum. Upon shifting the cultures to the permissive temperature, 32°C, in the presence of dinitrophenol, which blocks vesicular transport, the viral glycoprotein proceeded to completely fill the sarcoplasmic reticulum. Thus, both the endoplasmic reticulum located at the terminal cisternae of the sarcoplasmic reticulum, and the entire endoplasmic and sarcoplasmic reticulum appeared to be continuous. Shifting the culture temperature from 39°C to 20°C, resulted in prominent perinuclear staining throughout the fibers, accompanied by the appearance of distinct bright dots between the nuclei. Electron microscopic immunoperoxidase labeling indicated that these bright structures represented the Golgi apparatus. When either the tsO45-infected or wild-type virus-infected fibers were incubated at 32°C, the viral glycoprotein showed a staining pattern that consisted of double rows of punctate fluorescence. Immunogold labeling showed that the viral glycoprotein was present in both the transverse tubules as well as the endoplasmic/sarcoplasmic reticulum endomembranes. In addition, extensive viral budding was observed in the transverse tubules. Metabolic labeling experiments revealed that only half of the glycoprotein was processed in the Golgi, and this processed form had become incorporated into the budding viral particles. Thus, the processed viral glycoprotein was targeted to the transverse tubules. The other half of the glycoprotein remained endoglycosidase H-sensitive, suggesting its retention in the endoplasmic/sarcoplasmic reticulum endomembranes.

Key words: Myofiber, Sarcoplasmic reticulum, Transverse tubule

INTRODUCTION

Muscle cells are produced by the fusion of mononucleated myoblasts, thereby forming multinucleated myotubes. During the fusion process, intracellular organelles reorganize and extensive differentiation occurs. The filamentous contractile apparatus develops together with a complex intracellular membrane network consisting of the sarcoplasmic reticulum (SR) and the transverse (T)-tubulus system (reviewed by Flucher, 1992). In developing myotubes, these are tubulovesicular structures with a longitudinal orientation. In adult muscle fibers, however, they are highly organized into transverse cross-striated structures.

The SR of skeletal muscle fibers contains ER components such as BiP, protein disulfide isomerase and calnexin, strongly suggesting a continuity between these two structures (Volpe et al., 1992; Villa et al., 1993). Certain ER components, such as the Ca²⁺-dependent ATPase, are probably transported directly from the ER to the SR, since they could not be found in the Golgi (Jorgensen et al., 1977). However, calsequestrin, a major protein of the SR lumen, was found in the Golgi, suggesting a transport route for this protein through the organelle (Thomas et al., 1989). Calsequestrin clearly traveled through the Golgi in transfected mononucleated cells (Raichman et al., 1995), while Ca²⁺-ATPase did not (Karin and Settle, 1992). These findings suggest that proteins are translocated from the ER to the SR either directly or via the Golgi. It is, however, not known where the ER is situated in the SR.

In cultured myotubes, the reorganization and synthesis of organelles is reflected by the protein transport pathways and maturation processes. Thus, in chick embryo muscle cultures, acetylcholine esterase is not secreted correctly and most of it remains Endo H-sensitive, indicating a lack of Golgi processing (Rotundo et al., 1989). Furthermore, a major transport route from the Golgi to the SR was observed. In L6 myoblast-derived myotubes a fraction of the Golgi stacks were disassembled, and did not process glycoproteins normally (Kellokumpu et al., 1995). Multinucleated myotubes derived from a C2C12 cell line seem to secrete glycoproteins efficiently (Yao and Kurachi, 1992). A number of studies have shown that transgene products are secreted in skeletal muscle (Barr and Leiden, 1991; Dhawan et al., 1991; Dai et al., 1992), but the efficiency of the secretion was not analyzed and it is not known whether the secretion products were processed in the muscle fibers. It is not known
for sure whether skeletal myofibers exhibit a functional Golgi and where it is localized. Jasmin et al. (1989, 1995) have reported that Golgi structures are found only in the vicinity of the neuromuscular junction but Ralston (1993), on the contrary, has found Golgi elements throughout the muscle fiber.

Here, we have analyzed organelle localization and glycoprotein trafficking and processing in isolated skeletal myofibers, using the vesicular stomatitis virus (VSV) as a tool. We show that the isolated myofibers support VSV infection. Using the thermosensitive VSV mutant, tsO45, whose glycoprotein (G protein) is blocked in the ER at the restrictive temperature, we localized the ER at the A-I borders. At lower temperatures, the mutant G protein had direct access from the ER sites to the whole SR. Surprisingly, only a fraction of the viral G protein matured normally in the Golgi although the latter was present around every nucleus throughout the muscle fibers. The processed form of the G protein was targeted to the T-tubules, but not to the sarcolemma.

MATERIALS AND METHODS

Isolation of myofibers

Flexor digitorum brevis muscles from the feet of adult Spraque-Dawley rats (3-4 months of age) were used. The isolation procedure was as described by Bekoff and Betz (1977) and Bischoff (1986), with modifications. Briefly, the muscle was removed and placed in minimum essential medium (MEM) (Gibco Laboratories, Grand Island, NY) containing antibiotics (isolation medium). The epimysium and visible tendons were dissected away under a dissection microscope. The muscles were incubated with rotation in isolation medium containing 10 mg/ml collagenase (Worthington Biochemical Corporation, NJ) for 2.5 hours at 37°C, in an atmosphere of 5% CO2. Muscle fibers were detached from tendons and from each other by repeated trituration with a wide-mouth pipette. The individual fibers were sedimented at 1 g for 10 minutes through a 10 cm column of isolation medium containing 10% inactivated horse serum, in a 15 ml conical tube. The sedimentation was repeated three times to reduce the amount of muscle debris and mononucleate cells. Fibers from one muscle were finally suspended in 1 ml of culture medium composed of the isolation medium supplemented with 20% controlled process serum replacement (CPSR2) (Sigma Chemical Co, St Louis, MO) and 1% horse serum (Gibco). Cell culture dishes (30 mm in diameter) were coated with Matrigel (Becton Dickinson Labware, Bedford, MA), diluted 1:5 in culture medium according to the instructions of the manufacturer. To attach the fibers to the Matrigel substratum, approximately 100 μl of the fiber suspension were spread on a dish and incubated for 2 hours at 37°C. Thereafter, 1 ml of the culture medium was added. Approximately 200-300 fibers attached to

![Fig. 1. VSV infects isolated, adult rat skeletal muscle fibers. The viral G protein was immunostained using FITC-labeled secondary antibodies for visualization. (A) Fibers were infected for 8 hours at a dose corresponding to 100 pfu/ml for BHK cells. A confocal plane at 3 μm depth from the surface is shown. Note the intense perinuclear and spot-like staining, together with cross-striation. (B) Fibers were infected at a low dose (5 pfu) for 10 hours. The infection remained localized. (C,D) Double-staining for the G protein (C) and calsequestrin (D) in a myofiber cryosection shows intense G protein staining at the fiber cortex and less intense staining in the core. Infection time was 10 hours. Identical confocal planes are shown. Bars, 10 μm.]()}
Protein trafficking in muscle fibers

Each dish, representing 50-70% of the total. The fraction of mononucleate cells accounted for 0.2-1% of the total number of nuclei. The fibers were used for experiments after a 4 or 12 hour attachment period. The difference between these attachment times was not observed to affect the results. All the fibers showed a positive reaction with a fast type myosin antibody, indicating that they originated from a fast muscle.

Viral infection

Wild-type VSV was of the Indiana serotype. The mutant VSV tsO45 was the clone tsO45-6 (Griffiths et al., 1985). Virus stocks were produced in BHK cells and plaque titrated on BHK cell monolayers (Matlin et al., 1983). Myofibers were infected by applying the virus to the fibers in MEM containing 0.1% bovine serum albumin. Adsorption was for 1.5 hours (100 μl/dish) at 37°C (wild-type virus), or 32°C (tsO45), whereafter the virus inoculum was removed and replaced with MEM containing 2% CPSR and 0.1% horse serum (1 ml). The myofibers infected with the wild-type VSV were grown at 37°C, while those infected with the mutant were incubated at 39°C.

Antibodies

The polyclonal antibodies against the VSV G protein and the monoclonal antibody against the VSV matrix protein have been described previously (Metsikkö et al., 1992). The polyclonal antibodies were preadsorbed with isolated, fixed and permeabilized rat flexor digitorum brevis myofibers. The monoclonal antibodies against calsequestrin and Ca²⁺-ATPase were purchased from Affinity Bioreagents (NJ). Monoclonal antibodies against fast type myosin and β-COP were obtained from Sigma. Monoclonal BiP antibody was a gift from Dr Stephen Fuller (EMBL). Antibody against ribophorin II (clone 3D1; Hortsch et al., 1986) was generously provided by Dr Sean Clark (UCLA). FITC- and Texas red-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories.

Immunofluorescence studies

Myofibers were fixed with 3% paraformaldehyde and permeabilized by Triton X-100 treatment (Louvard, 1980). For double immunofluorescence studies, polyclonal and monoclonal antibodies were applied to the cells at 1:200 dilution and incubated for 60 minutes at 37°C. The cells were then washed twice for 30 minutes with PBS, and secondary antibodies labeled with FITC, or Texas red, were applied at appropriate dilutions and incubated for 1 hour at 37°C. Nuclei were visualized with Hoechst 33258 stain. Embedding was performed with Mowiol 4-88 (Hoechst, Frankfurt, Germany) containing 2.5% 1,4-diazobicyclooctane (Sigma). Samples were examined using a laser confocal microscope (Leica Lasertechnik, Heidelberg, Germany) equipped with a multiline 750 mW Omnichrome argon-krypton laser (Chino, CA). The confocal system was built around a Leitz Aristoplan microscope (Oberkochen, Germany).

Under the incubation conditions used, the penetration of the antibodies into the muscle fibers was restricted. Therefore, fibers were fixed with paraformaldehyde, scraped together and frozen in isopentane pre-chilled in liquid nitrogen. Frozen sections (4 μm) were cut using a cryomicrotome. The sections were collected on glass slides, permeabilized and immunostained as above.

Electron microscopy

For thin-section electron microscopy, myofibers were fixed with 2.5%
glutaraldehyde. Samples were postfixed with 4% OsO₄, dehydrated and embedded in Epon. For immunoperoxidase labeling, fibers were fixed with 3% paraformaldehyde for 20 minutes, followed by 0.5% saponin treatment. The fibers were blocked with fetal calf serum and then incubated with pre-adsorbed anti-VSV G protein antibodies, followed by incubation with secondary antibodies conjugated to peroxidase. These incubations were carried out in the presence of saponin. The fibers were fixed with 2.5% glutaraldehyde before the diaminobenzidine reaction. Sections (100 nm) were examined with the Philips 410 electron microscope. For immuno-gold labeling, fibers were fixed for 30 minutes with 6% paraformaldehyde containing 0.1% glutaraldehyde and cryoprotected with 2.3 M sucrose for 10 minutes before cryosectioning. The sections (100 nm) were blocked with fetal calf serum and then incubated with anti-VSV G protein antibodies, followed by incubation with Protein A conjugated to 10 nm gold particles. Background was evaluated using noninfected fibers.

**Metabolic labeling**

The fibers were allowed to attach for 4 hours prior to infection. Pulse-chase labeling with [³⁵S]methionine (Amersham Corp., Bucks, England) was performed at 10 hour post-infection. A 15 minute pulse labeling (50 μCi/ml) was performed, followed by various chase times. Overnight labeling (10 hours) was performed in MEM containing 5 μM methionine, using 100 μCi/ml of [³⁵S]methionine.

**Other methods**

In pulse-chase experiments, fibers were solubilized with PBS containing 1% Triton X-100 and 1% deoxycholate. The viral G protein was immunoprecipitated using specific antibodies and immobilized Protein A (Sigma). Endo H (Boehringer, Mannheim, Germany) digestion was performed as described by Bennett et al. (1988). Cell surface biotinylation was performed after [³⁵S]methionine pulse labeling and a 90 minute chase at 0°C, as described by Lisanti et al. (1988). The biotinylated proteins were purified by immobilized streptavidin (Boehringer) according to the method of Hare and Lee (1989).

SDS-PAGE was performed as described by Laemmli (1970). Dried gels were exposed to Fuji RX film. Quantitation of radio-labeled bands was performed with a Molecular Dynamics PhosphorImager SI, using the GelQuaNT program.

**RESULTS**

**Isolated myofibers support VSV infection**

Electron microscopic analysis showed that the isolated myofibers were devoid of basement membrane and, therefore, should be susceptible to viral infection. To evaluate whether the fibers supported infection, immunofluorescence staining was performed on permeabilized cells. When high viral doses (100 pfu/cell; plaque titration was performed using BHK 21 cells) were used, the G protein and the matrix protein were uniformly distributed over the myofibers after a 6 hour infection period. [³⁵S]methionine-labeling experiments showed that, at this time point, cellular protein synthesis was still going on, but was practically abolished at 10 hours post-infection. The intensity of the G

![Fig. 3. The mutant G protein moves into the longitudinal SR upon shifting from the restrictive, to the permissive temperature. The fibers were infected for 6 hours at 39°C and then for 5 minutes at 32°C, and a further 60 minutes in the presence of 1 mM dinitrophenol. (A) G protein localization at 39°C; (B) double-staining for BiP; (C) G protein localization after dinitrophenol treatment at 32°C, and double-staining for BiP (D). G protein was visualized with FITC- and BiP with Texas red-conjugated secondary antibodies. Broken lines mark two adjacent Z lines. Bars, 10 μm.]
protein fluorescence varied from fiber to fiber at all time points studied. No staining was seen in non-infected cells. The G protein appeared as prominent, discontinuous rings around every nucleus and as bright spots between the nuclei (Fig. 1A). A uniform, cross-striated staining pattern was also seen. At lower doses, the infection remained localized (Fig. 1B).

Since the antibodies did not penetrate into the interior of fixed, whole myofibers, frozen sections were processed for immunofluorescence staining. Such analyses showed that the VSV G protein was also present in the interior regions of the myofibers. However, the most intense labeling was constantly seen in the region beneath the sarcolemma (Fig. 1C). Double staining for calsequestrin exhibited a very similar staining pattern (Fig. 1D).

**The tsO45 G protein localizes to the ER at the A-I junctions**

We utilized the temperature-sensitive mutant, VSV tsO45, to analyze the location of the rough ER compartment in muscle fibers. The G protein of this mutant is retained in the ER at the restrictive temperature, 39°C (Schnitzer et al., 1979; Zil-
berstein et al., 1980), because it does not fold correctly (Hammond and Helenius, 1994). When tsO45-infected myofibers were grown at 39°C, the mutant G protein was seen as spots located at A-I junctions (Fig. 2A). A perinuclear component was also present. The spots usually existed as doublets (Fig. 2C), rather than as the single spots seen with calsequestrin (Fig. 2B). A monoclonal antibody against BiP (see Fig. 3B,D) also stained the A-I junctional areas, leaving the Z- and the M-lines clear. An identical result was obtained with an antibody against ribophorin II (not shown) which is a marker of the rough ER. The fluorescence signal with these antibodies was weak, and we could not resolve whether the staining pattern was composed of doublets. These results suggest, however, that the ER was localized to the terminal cisternae membranes of the SR. Immunogold localization studies were also performed for the tsO45-infected cells grown at 39°C. Such studies verified the localization of the mutant G protein in the terminal cisternae of the SR (Fig. 2D).

We next analyzed whether the mutant G protein was able to translocate directly from the ER to the SR. The temperature was thus shifted from 39°C to 32°C, under conditions where vesicular budding from the ER was blocked. The fibers were first incubated at 32°C for five minutes to allow folding of the G protein. A vesicular budding inhibitor, dinitrophenol (1 mM) (Copeland et al., 1988), was then added for a period of 1 hour at 32°C. After the 1 hour incubation, the original A-I staining pattern (Fig. 3A) changed so that the G protein was found over the Z line (Fig. 3C), resembling the staining pattern of Ca²⁺-ATPase (not shown) which is a marker of the longitudinal portions of the SR (Jorgensen et al., 1979). The staining pattern of BiP remained unchanged during the incubation with dinitrophenol. A similar result was obtained when the myofibers were shifted from 39°C to 15°C for 2 hours, suggesting that the 15°C compartment (Saraste and Kuismanen, 1984) located near or within the ER/SR endomembrane system.

The tsO45 G protein moves into perinuclear Golgi compartments at 20°C

At 20°C, a transport block results in the accumulation of exocytic proteins in the trans-Golgi network (Griffiths et al., 1985). The temperature was therefore shifted from 39°C to 20°C, for 2 hours, in the presence of cycloheximide, to inhibit protein synthesis. Immunofluorescence staining showed that the G protein moved into a compartment that consisted of perinuclear ring structures and bright spots between the nuclei (Fig. 4A,B). The perinuclear ring structures were found around every nucleus, visualized by Hoechst stain. This staining pattern was characteristic of the Golgi pattern in myotubes (Tassin et al., 1985; Metsikkö et al., 1992). When the fibers were incubated at 15°C, at which temperature transport to the Golgi should be blocked, no such staining appeared. Electron microscopic immunoperoxidase labeling after the 2 hour incubation period at 20°C indicated staining of Golgi elements around nuclei and between sarcomers throughout the myofiber (Fig. 4C,D), providing firm evidence that the perinuclear staining pattern and the bright spots seen in Fig. 4A and B represented myofiber Golgi elements. Furthermore, we found that the perinuclear ring structures that were seen with the mutant virus during the 20°C incubation and also during a wild-type VSV infection, colocalized with coatomer (β-COP) staining which marks the Golgi in fibroblasts (Allan and Kreis, 1986). Fig. 5 shows the colocalization of the perinuclear components of the VSV G protein and β-COP.

VSV G protein is targeted to the T-tubules in myofibers

When the tsO45 virus was grown at the permissive temperature, 32°C, or when wild-type VSV was used, an intense cross-striated staining pattern of the G protein was seen, together
with a perinuclear staining pattern. The cross-striated staining pattern consisted of double rows of fluorescent spots regularly distributed on both sides of the Z line, shown in Fig. 6A. No doublet structure such as was seen with the tsO45 at 39°C could be discerned but a faint fluorescence was often seen between the double rows over the Z line (not shown). The double row pattern of the VSV G protein staining resembled that described for triad components (Yuan et al., 1991) and calsequestrin (Jorgensen et al., 1977). Double-staining for the VSV G protein and calsequestrin showed a colocalization of the double rows (Fig. 6A,B), suggesting that the G protein was concentrated in triad areas.

In non-permeabilized cells, a faint granular G protein staining could be detected at 6 hours post-infection (not shown), while after a 10 hour infection period, the staining was seen as coarse patches. Similar results were obtained when unfixed cells were processed for immunofluorescence staining at 0°C, shown in Fig. 6C. The patches were arranged in a cross-striated pattern at the A-I borders where T-tubules protrude into the plasma membrane, suggesting that the G protein localized to the T-tubules.

To analyse whether the G protein was initially inserted into a cross-striated dot pattern that possibly corresponds to the T-tubular openings at the cell surface, we added anti-G protein antibodies to the medium at 6 hours post-infection. The myofibers were then washed and fixed after a 2, 5 or 8 hour incubation period with the antibodies. The bound anti-G protein antibodies were visualized by fluorescein-conjugated secondary antibodies. We found dot-like staining pattern at all time points analysed. A majority of the dots localized in double rows on both sides of the Z lines (Fig. 6D), where the T-tubules protrude.

Immunogold labeling studies showed the VSV G protein to be present in the T-tubules as well as in the SR (Fig. 7). Many of the gold particles seen within the myofibers decorated virions, however. When plastic sections were examined by electron microscopy, extensive viral budding was seen in the T-tubules at 10 hours post-infection and later. Viral budding was seen at the plasma membrane, too, but exclusively at the A-I junctions where the T-tubules protrude. Furthermore, the A-I areas of the plasma membrane often contained aggregates of viral particles. Fig. 8 shows examples of virus budding in muscle fibers. We conclude that in skeletal myofibers VSV G protein is targeted to the T-tubules.

Only a fraction of the VSV G protein acquires Endo H-resistance

In fibroblasts, the VSV G protein matures during transport through the trans-Golgi, into a form that exhibits slower mobility when analyzed by SDS-PAGE (Kornfeld and Kornfeld, 1985; Balch and Keller, 1986). To investigate whether a similar maturation occurred in skeletal myofibers, we performed pulse-chase labeling with [35S]methionine. Fig.

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**Fig. 6.** Intracellular and surface localization of the VSV G protein in myofibers. (A and B) Double-staining for the VSV G protein (A) and calsequestrin (B) in an infected myofiber at 8 hours post-infection. Identical confocal planes are shown. The G protein was visualized by FITC-conjugated secondary antibodies, while Texas red-conjugated anti-mouse IgG was used to visualize calsequestrin. N, a nucleus. (C and D) Surface localization of the VSV G protein. Live cells were immunostained at 0°C without fixation, and a coarse cross-striated staining pattern appeared (C). When anti-G protein antibodies were applied to the medium at 6 hours post-infection and the fibers were then fixed at 8 hours post-infection, followed by incubation with FITC-conjugated secondary antibodies, a staining pattern showing rows of dots at the A-I junctions was seen (D). Arrows mark the positions of two adjacent Z lines. Bars, 10 μm.
9A shows that the maturation of the wild-type G protein was defective in the myofibers. Only 46% of the total labeled G protein was transformed into a less mobile form during a 180 minute chase period (Fig. 9B). Similar results were obtained when the labeling was performed at 6 hours post-infection, when cellular protein synthesis was still going on (not shown). We constantly found that the G protein band that was not transformed to the less mobile form was processed into a slightly more mobile form during the chase. Similar defective maturation of the G protein was observed with tsO45-infected myofibers grown at 32°C.

The conversion of the G protein from an Endo H-sensitive form into an Endo H-resistant form is a Golgi-specific event (Davidson and Balch, 1993). The Endo H-sensitive and resistant forms can be distinguished by their mobilities in SDS-PAGE gels (Dunphy et al., 1985; Balch and Keller, 1986). In myofibers, the more mobile G protein form remained Endo H-sensitive during the chase, while the slower form became resistant to the enzyme (Fig. 9C). It can be concluded that the faster form was not processed in the medial Golgi. When tsO45-infected cells were pulsed at 39°C and then chased at 39°C, the amount of the G protein decreased, with a half time of about 2 hours, suggesting degradation (Fig. 10A). When the chase was performed either at 39°C, or 15°C, the G protein remained totally Endo-H sensitive and no mobility shifts occurred (Fig. 10B).

The finding that the more mobile G protein remained Endo H-sensitive suggests that it was retained in the ER/SR endomembrane system, possibly due to a folding defect. Treatment of cells with dithiothreitol has been shown to be an indicator of disulfide bridge formation and folding (Braakman et al., 1992; Hammond and Helenius, 1994). We therefore analyzed whether the G protein became resistant to dithiothreitol during the chase. Fig. 11 shows that the G protein was totally unaffected by this treatment. It thus seems that proper folding occurred.

**Mature G protein appears in the viral particles**

Plaque assays of the conditioning medium of the myofibers at 10 hours post-infection, using wild-type VSV, indicated a small amount of infective particles (9×10⁵ pfu/ml). To analyze which form of the G protein appeared in the virions, we infected myofibers for 6 hours and then labeled the fibers with [³⁵S]methionine for 10 hours. The labeling medium was subjected to sucrose gradient centrifugation to purify the virions. SDS-PAGE showed that the virions contained the
mature form of the G protein (Fig. 12). About 6% of the total G protein was recovered in the form of virions. Cell surface biotinylation experiments, performed after pulse-labeling and a 180 minute chase period, also detected a faint band of the mature form of the G protein (not shown).

**DISCUSSION**

We employed enveloped viruses to analyze protein transport in isolated, multinucleate muscle cells. The results show that rat myofibers were readily infected with VSV after removal of the basement membrane. At low doses, the infection remained localized, as occurs with myotubes (Metsikkö et al., 1992), while at high virus doses, the infection occupied the whole length of the myofibers. We found that other viruses, such as Semliki Forest virus, and the Influenza virus, could also infect myofibers (unpublished).

ER-retention signal-containing chaperones, such as BiP and calnexin, have been reported to be present in the whole SR of skeletal muscle fibers (Volpe et al., 1992; Villa et al., 1993). Where the rough ER is situated in the ER/SR endomembrane network has remained obscure. When we used the tsO45 mutant at the restrictive temperature, A-I junctions were labeled (Fig. 2). An antibody against BiP also labeled the A-I junctions, suggesting localization to the terminal cisternae but not to the whole SR. On shifting to the permissive temperature but with budding from the ER blocked, we saw movement of the G protein to the longitudinal portions of the SR (Fig. 3). This finding is compatible with the proposal that ER and SR membranes are continuous (Volpe et al. 1992). However, our results show that the ER and the SR are not identical. We do not know why the mutant G protein remained immobile in the myofibers at 39°C, since the mutant G protein rapidly moved to the smooth ER at 39°C in UT-1 cells (Bergman and Fusco, 1990). However, Hammond and Helenius (1994) showed that

**Fig. 8.** Budding and accumulation of VSV particles in the A-I junctional areas on the plasma membrane (A) and in T-tubules (B). Plastic sections (100 nm) were analysed by electron microscopy. Bars, 1 μm (insets, 100 nm).
the unfolded G protein has a tendency to aggregate, which probably immobilizes it. Another possibility is that in its unfolded form the G protein was bound to BiP which obviously located at the A-I junctions. At lower temperatures, the intracellular G protein has been shown to be freely mobile (Scullion et al., 1987; Storrie et al., 1994).

The mutant G protein moved into perinuclear and dispersed structures at 20°C (Fig. 4). Our electron microscopic immunoperoxidase labeling studies showed that at 20°C the G protein located in Golgi structures. This kind of Golgi localization in muscle fiber is fully compatible with the results of Ralston (1993), who, by detailed morphological studies, showed the presence of Golgi stacks around the nuclei and throughout the periphery of myofibers. Our study shows further that there is a transport route from the ER into all the Golgi units. Interestingly, we found that the nuclei located beneath the neuromuscular junctions often exhibited a very prominent Golgi staining (not shown). This finding parallels that of Jasmin et al. (1989, 1995), but we do not agree that Golgi elements exist exclusively in the end-plate region.

Analysis by confocal laser scanning microscopy showed that the VSV G protein was present in all sarcomers (Fig. 6) and

Fig. 10. The tsO45 G protein is not processed when transport to the Golgi is blocked. TsO45-infected cells were labeled with [35S] methionine at 39°C for 15 minutes and then chased. The G protein was immunoprecipitated and subjected to SDS-PAGE analysis. (A) The amount of the G protein decreased with time but no maturation occurred when the chase was performed at 39°C. (B) The G protein remained Endo H-sensitive when chased for 90 minutes at either 39°C or 15°C. G, the core-glycosylated G protein.

Fig. 11. VSV G protein is insensitive to treatment with dithiothreitol in myofibers. The fibers were infected for 10 hours and then pulse-labeled with [35S] methionine, followed by no chase or a 90 minutes chase. Some of the fibers were incubated for 15 minutes in the presence of 5 mM dithiothreitol and then processed for SDS-PAGE under non-reducing conditions. After the chase, the immature form of the G protein (G_i) was not affected, and no G protein was detected at the position of the reduced G protein band (arrow), indicating that dithiothreitol did not disassemble the disulfide bridges of the G protein. Most of the G protein regularly disappeared when the dithiothreitol-treatment was performed immediately after the pulse. N, VSV N protein.

Fig. 12. Mature G protein appears in the virions. Infected myofibers were labeled for 10 hours with [35S] methionine. Pre-cleared medium was subjected to centrifugation in a 5-20% sucrose gradient, in a Beckman SW 40 rotor at 20,000 rpm for 45 minutes (Metsikkö and Garoff, 1989). The VSV peak was collected and virions were pelleted at 25,000 g for 1 hour, and analyzed by SDS-PAGE. The mature form of the G protein (G_m) was recovered in the virions while both the mature and the immature (G_i) forms were present in the immunoprecipitate of the myofiber lysate.
免疫金染色定位了G蛋白在T-小管中的位置。该模型假定ER和SR膜是连续的。原本的G蛋白在T-小管中的位置是不变的。因此，整个G蛋白未能在ER中稳定。G蛋白可以被运输到SR，而不能在ER中运输。G蛋白在ER/SR系统中的成熟过程发生在早期感染，而且在不成熟的G蛋白中观察到这种现象。成熟的G蛋白被运输到T-小管系统。

![Fig. 13. Plausible transport routes of the VSV G protein in skeletal myofibers. The model postulates that the ER and the SR membranes are continuous.](image)

We could show that virions collected from the medium possessed the mature form of the G protein. Our electron microscopic data strongly suggest that these virions originated from the T-tubules and their orifices. Furthermore, immunofluorescence staining of non-permeabilized cells revealed rows of dots at the A-I junctions, leaving other areas of the plasma membrane clear. Similar results were obtained when primary antibodies were added to the medium during the viral infection. Therefore, we conclude that the mature form of the G protein was targeted to the T-tubules and was absent at the sarcolemma. It has been shown earlier that T-tubular proteins differ from those of the plasma membrane throughout muscle development (Yuan et al., 1990; Flucher et al., 1991). A 50 kDa sarcolemmal protein was not present in T-tubules, and a 28 kDa T-tubular protein was not found at the sarcolemma (Jorgensen et al., 1990). While our results indicate a pathway from the Golgi to the T-tubules, it remains unresolved how sarcolemmal proteins are targeted.

In conclusion, our results locate the ER at the A-I junctional area of the SR membrane system and confirm the view (Ralston, 1993) that Golgi elements are found throughout the myofibers. Furthermore, they show that skeletal myofibers process a fraction of the VSV G protein and target the processed form to the T-tubulus system. The sarcolemma received little, if any, G protein. A large fraction of the G protein remained Endo H-sensitive, suggesting that it was not transported beyond the cis-Golgi and that it was retained in the ER/SR endomembrane system. Fig. 13 depicts the hypothetical transport routes for the VSV G protein in muscle fibers.

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**REFERENCES**


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