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INTRACELLULAR TRANSPORT OF VSV G PROTEIN OCCURS IN CELLS LACKING A NUCLEAR ENVELOPE

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SUMMARY: The intracellular transport of the G protein of a temperature sensitive vesicular stomatitis virus (VSV) mutant (ts 045) was examined in nucleated chinese hamster ovary (CHO) cells and in CHO cells subjected to enucleation with cytochalasin B. Although protein synthesis was virtually unaffected, enucleated cells synthesized only 30-45% of the amount of G protein assembled in control cells. As measured by acquisition of endoglycosidase H resistance, the rate of transport of the G protein from the RER to the medial golgi was found to be similar for nucleated and enucleated cells ($t_{\frac{1}{2}}$ = 15 min). These data suggest that elements of the RER may directly transfer their contents to the Golgi, without the obligatory involvement of an intact NE.

The nuclear envelope is often considered to be a specialized region of the RER; indeed, the NE shares many of the enzyme activities of the endoplasmic reticulum (1). Like the RER, the NE is a site of membrane glycoprotein synthesis (2) and free diffusion of plasma membrane glycoproteins has been demonstrated to and from the inner nuclear membrane (3). Recently, work in this laboratory and others has demonstrated that polypeptides of the nuclear pore complex are glycoproteins containing O-linked N-acetylglucosamine (4-7). In addition, an integral membrane glycoprotein has been localized to the region of the pore complex (8).

A number of studies have suggested that the NE may play a role in membrane glycoprotein synthesis and transport. Nuclear envelope fragmentation coincides with a temporary cessation in the processes of glycosaminoglycan synthesis, endocytosis, secretion and recycling (9-11). Newly formed endoplasmic reticulum emerges from the nuclear envelope in cells overproducing HMG-Co A reductase (12). In another study, a variant of the Rous Sarcoma Virus *env* glycoprotein which lacked the cytoplasmic and transmembrane domains was shown to accumulate in the endoplasmic reticulum; the protein was excluded from the NE and Golgi apparatus (13). The vesicular stomatitis virus (VSV) G protein has been shown to be synthesized in elements of the RER and in the nuclear envelope of cultured cells. During VSV G protein intracellular transport, blebbed sites were observed on the nuclear envelope where G-protein was thought to be concentrated in the early stages after synthesis and prior to delivery to the Golgi apparatus (14). Taken together,

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these observations suggest a possible role of the nuclear envelope in glycoprotein assembly and intracellular transport.

Techniques have been developed which allow the selective removal of the nucleus and associated nuclear envelope from living cells by cytochalasin B-induced enucleation (15-19). Such enucleated cells have mitochondria, Golgi apparatus, lysosomes, ribosomes, and endoplasmic reticulum; they lack only the nucleus and nuclear envelope (20-23). Here, the intracellular transport of the G protein of a temperature sensitive mutant of VSV was examined in enucleated cells to determine whether the nuclear envelope is required for transport to the Golgi apparatus. The results suggest that the rate and extent of this intracellular transport process are unaltered by removal of the nucleus and nuclear membrane.

METHODS

Cell Culture and Enucleation

BHK-21 and VERO cells were cultured in 75-ml flasks (Costar, Cambridge, NA) with Dulbecco's Modified Eagle Medium, supplemented with 10% Fetal Calf Serum (GIBCO, heat inactivated), 1% Glutamine (GIBCO, Long Island, NY), and 1 % penicillin-streptomycin (GIBCO, penicillin-5000 U/mlg, streptomycin-5000 mcg/ml). CHO 10900 cells were cultured in α-Modified Minimal Essential Medium, 10% Fetal Calf Serum, 1% Glutamine, and 1% penicillin-streptomycin. The enucleation procedure was adapted from previous methods (17,18) CHO cells at 50% ($1x10^5$ cells) confluence in 60 mm tissue culture dishes were infected with VSV ($3x10^7$ pfu/5 ml) for 1 hour at 37°C. Centrifuge bottles with the top removed were filled with 125 mL of serum-cultured containing medium with cytochalasin B at 5 µg/mL. The filled bottles were pre-incubated at 37°C for 10 minutes before cells were added. Dishes were placed in an inverted position in the bottles, incubated for 10 minutes in a 40°C bath, spun at 12,000 x g for 40 minutes at 40° C and washed with medium. To determine the extent of enucleation, dishes were stained with daunomycin, (50 µg/mL)(Sigma).

Antibodies

Polyclonal anti VSV G-protein antibodies were produced in rabbits and were a generous gift from Dr. Richard Schlegel. The first antibody (anti-G and N/NS) was prepared by immunization of a rabbit with octyl-B-D glucopyranoside extracted VSV. The second antibody (anti-IKL) was prepared by immunization of a rabbit with a peptide corresponding to the last 20 amino acids of the G protein (beginning Ile-Lys-Leu. . . $_{20}$). The immunoglobulin was affinity purified using the peptide coupled to cyanogen bromide activated Sepharose 4B. Goat anti-rabbit IgG coupled to fluorescein and rhodamine were obtained from Jackson Laboratories.

Viral Infection, Transport, and Immunofluorescent localization

Plaque purified VSV ts 045 had a titer estimated at $3x10^8$ pfu/mL at 32° C and $4X10^2$ at 40° C. Cells were infected with VSV ts 045 at high multiplicity (100 pfu/cell), incubated for 1 hour at 37°C and washed with fresh medium. After incubation at 4 hours at 40° C, the experimental dishes were filled with 32°C medium and placed at 32°C, while parallel control dishes were maintained at 40°C. Cells were fixed after various time points with 3.7% Formalin (Fisher 37% w/v) in PBS (GIBCO Dulbecco's phosphate-buffered saline + Ca^{2+} + Mg^{2+}). The monolayers were processed for indirect immunofluorescence as previously described (6). When wheat germ agglutinin (WGA) was used, the lectin was added to attain a final concentration of 100 µg/mL in a 30 minute incubation period. The cells were then washed and mounted as described above for indirect immunofluorescence.

Metabolic labelling and immunoprecipitation

Infected monolayers (either cytoplasts or whole cells) were washed with PBS (maintained at 40° C) and supplemented with methionine free alpha modified eagles medium (alpha MEM-methionine) containing ^{35}S-methionine (200 µCi/2ml). After labelling at 40° C for 3 hours, the monolayers were washed with 40° C MEM (3 times). MEM containing 10 µg/ml cycloheximide and 10 mM unlabelled methionine (at

32° C) was added and after various time intervals, the G protein was extracted by incubation for 10 minutes at 4° C with ice cold buffer containing 0.1 M NaCl, 25mM Tris HCl, pH 7.5, 2.0mM ethylenediaminetetraaceticacid (EDTA), 0.5% Triton X-100 and 0.5% Sodium cholate (G protein buffer). After clarification by centrifugation at 100,000 X g for 30 minutes, aliquots were incubated with the appropriate dilution of purified antibody or antiserum overnight at 4 ° C. Staphylococcus aureus cells (50 microliters of 10% suspension) were added and incubated for 1 hour at 4 °C The pellets were then washed 4 times with G protein buffer and resuspended in a buffer containing 50 mM Sodium citrate pH 5.5, and 0.1% Sodium dodecyl sulfate (endoglycosidase H buffer). Aliquots were either supplemented with endoglycosidase H (Miles laboratories) to a final concentration of 10 mUnits/ml or with buffer alone. Following incubation at 37 ° C for 12 hours, the reaction was terminated by the addition of an equal volume of 2X gel loading buffer and the mixture was subjected to SDS-PAGE on a 8% SDS gel. SDS-PAGE and autoradiography was carried out as described previously (24).

Protein synthesis

The rate of protein synthesis was measured by incubating enucleated or control monolayers with ^{35}S-methionine (100 µCi/ml) for 0, 1,2,3, and 4 hours at 37° C. At the indicated time periods, the cells were washed with ice cold PBS, scraped from the plate, and sedimented at 3000 X g. The resulting cell pellet was treated with 10 % Trichloroacetic acid (TCA) for 10 minutes at room temperature, washed four times and neutralized with sodium hydroxide. Methionine incorporation was monitored by liquid scintillation counting. An aliquot was also taken for measurement of protein content. Protein assay was performed by the Pierce protein assay (BCA protein assay; Pierce Chemical company).

RESULTS AND DISCUSSION

The antibodies used in this study were first evaluated for their ability to immunoadsorb G protein from ^{35}S-methionine labelled Vero cells previously infected with VSV ts 045. Vero cells were infected with a high multiplicity of VSV ts O-45 and labelled for 3 hours at 40° C. At this temperature, the G protein of VSV ts 045 accumulates in the rough endoplasmic reticulum and nuclear envelope and the oligosaccharide would remain sensitive to the enzyme endoglycosidase H. As shown in Figure 1 A, polyclonal antisera produced against partially purifed VSV G protein recognizes two polypeptides corresponding in molecular mass to the G protein and the N/NS proteins of VSV. The polypeptides are not immunoprecipitated from uninfected cells (data not shown) or from cell extracts immunadsorbed using pre-immune serum (Figure 1 E and F). The apparent molecular mass of the G protein was altered by incubation with the enzyme endoglycosidase H. The immunoprecipitated N/NS proteins, which are not glycoproteins, serve as a negative control and were not altered in their mobility. Antibodies made against a synthetic peptide corresponding to the carboxyl terminal amino acids of VSV G (anti-IKL) also recognized VSV G (Figure 1 C and D). Both antisera detected the G protein in infected cells analyzed by western blotting (Data not shown).

CHO cells were used for enucleation experiments since these cells were found to be quite susceptible to cytochalasin B-induced enucleation. The temperature sensitive transport of the G protein of VSV ts O-45 in CHO cells was first followed by indirect immunofluorescence using anti-IKL antibody directed against the carboxyl terminal of VSV G (Figure 2 top panels). At O time and after 1 min at the permissive temperature, the pattern of immunofluorescence observed was consistent

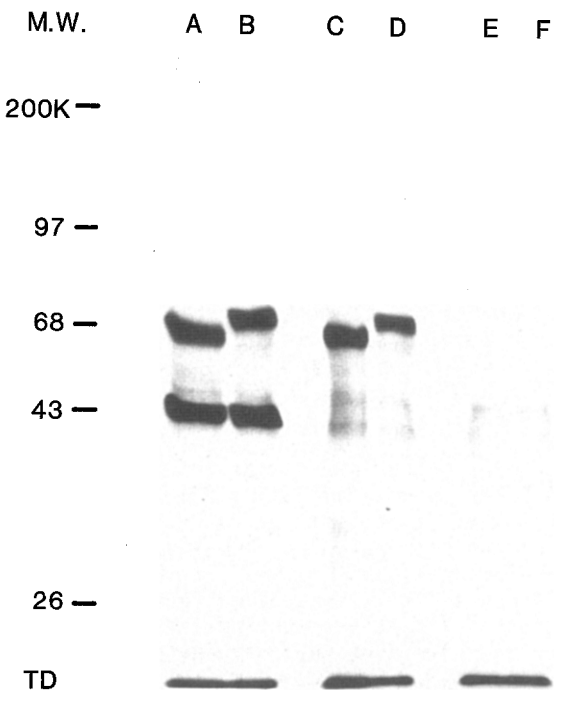

Figure 1. Characterization of Antisera recognizing VSV ts O-45 G protein
VSV ts O-45 infected Vero cell extracts were immunadsorbed using polyclonal anti-G and N/NS proteins (lanes A and B), anti-IKL (lanes C and D) or with Normal Rabbit IgG (lanes E and F). In lanes A, C, and E, the immunoprecipitates were incubated at 37° C for 12 hours with Endoglycosidase H.

with the presence of the G protein in elements of the RER and the nuclear envelope. After 10 minutes, G protein began to accumulate in a perinuclear organelle with the morphological characteristics of the golgi apparatus. These kinetics were not significantly altered by the concentrations of cytochalasin B used for enucleation (Fig 2 bottom panels). Conditions were developed which would allow the removal of nuclei from the cells by cytochalasin induced enucleation. To assess enucleation quantitatively, the fluorescent compound daunomycin, which selectively labels nuclei was used. The absence of intact nuclear envelopes in the cytoplasts was confirmed by staining with an anti-lamin A and C monoclonal antibody. Although untreated CHO cells exhibit perinuclear staining with this antibody, the CHO cytoplasts were devoid of label (Data not shown).To ensure that the cytoplasts still retained the golgi apparatus after enucleation, CHO cells and cytoplasts were stained with fluorescently labelled WGA to visualize this organelle. As shown in Figure 3, the cytoplasts retain an organelle which stains brightly with WGA even after removal of the nucleus.

VSV ts 045 infected CHO cells were subjected to enucleation using cytochalasin B followed by centrifugation. The resulting cytoplasts and control cells (not subjected to centrifugation) were metabolically labelled with ^{35}S-methionine for 3 hours at 39°C. In 4 separate experiments, the amount of methionine labelled G protein in the cytoplasts ranged from 30% to 45% of that in control

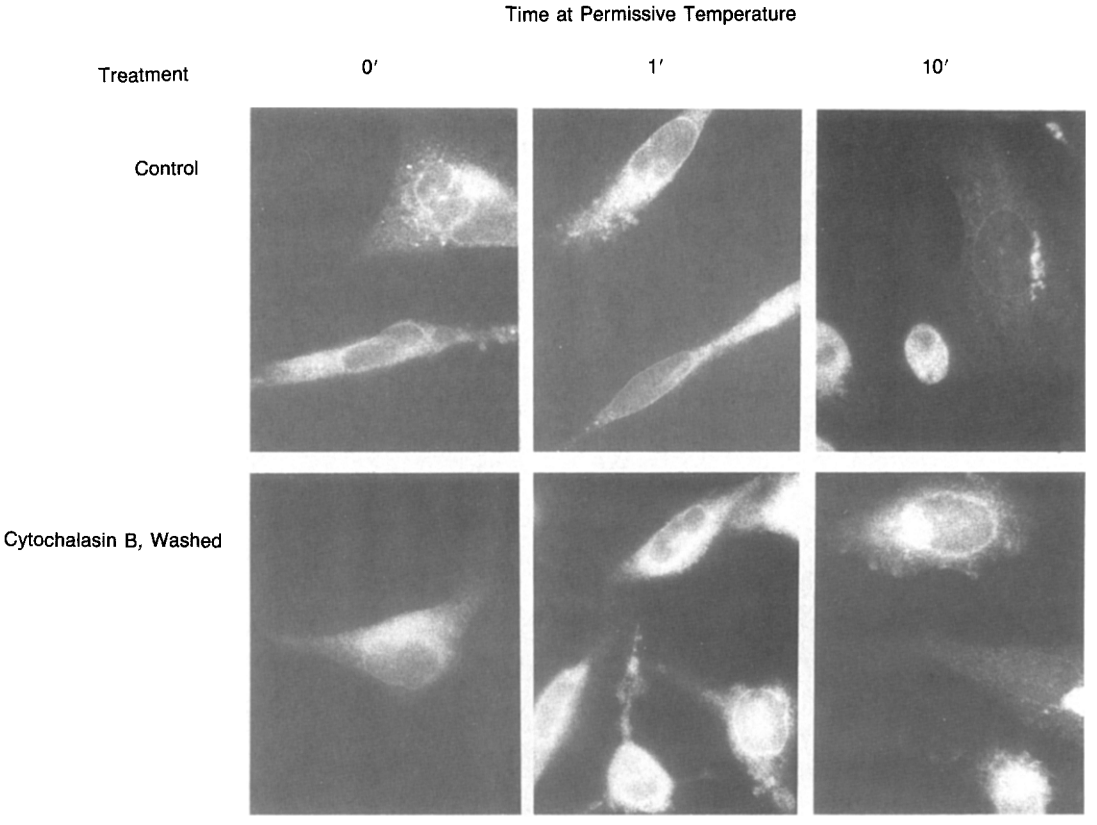

Figure 2. Transport of VSV ts O-45 G protein from RER to Golgi in Control and cytochalasin B treated CHO cells

Cytochalasin B treated and control CHO cells were infected with VSV ts O-45 for 1 hour at 37° C, washed and incubated at 40° C for an additional 3 hours. The temperature was then shifted to 32° C for 0, 1 or 10 minutes and the location of VSV G protein was examined using anti-IKL rabbit antibody.

cells. Significantly, the rate of total protein synthesis, measured as methionine incorporation per mg protein, was reduced only 5-12% under these conditions. These findings are consistent with the study of Puddington (2) who found that a large amount of newly synthesized G protein could be found on nuclei isolated from a variety of infected tissue culture cells. To assess the effects of enucleation on transport to the Golgi apparatus, infected cells were subjected to enucleation or treated with cytochalasin B without centrifugation. The temperature was lowered to 32°C for various periods of time prior to solubilization of the G protein and immunoprecipitation. The rate at which the G protein acquired resistance to endoglycosidase H was then determined. Sensitivity to the endoglycosidase was examined by the altered mobility of the immunoprecipitated G protein on SDS-PAGE

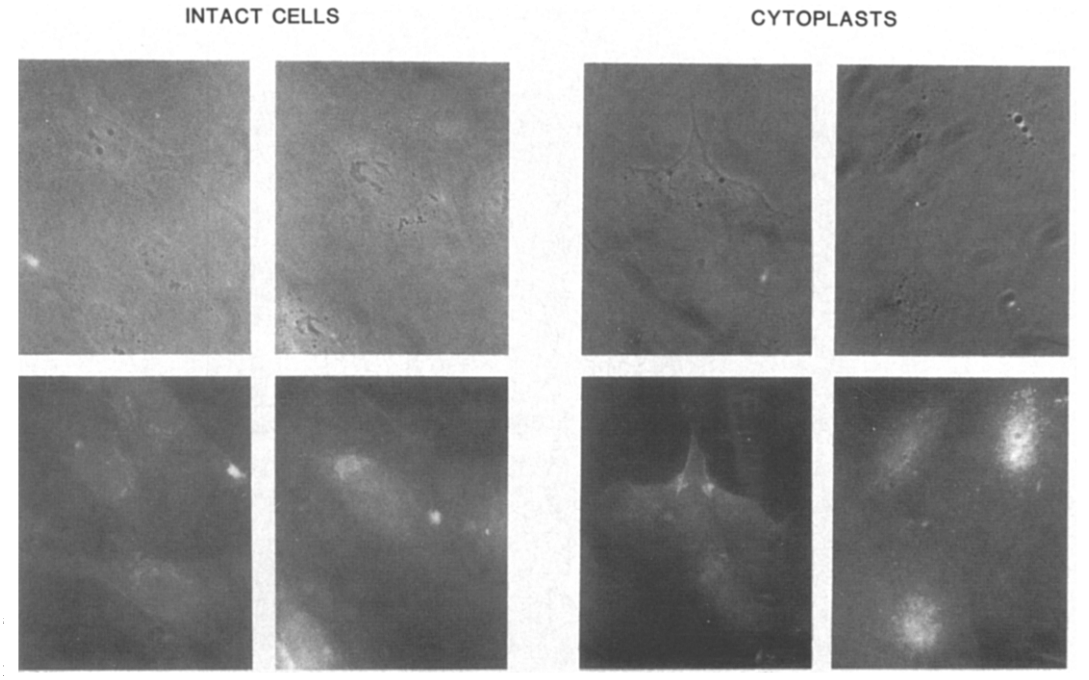

Figure 3. Nucleated and Cytochalasin-induced Enucleate cells (Cytoplasts) stained with wheat germ agglutinin

CHO cells were subjected to cytochalasin B-induced enucleation as described in "Experimental Procedures." These cells and a dish of cells carried through a mock enucleation were fixed with 3.7% formaldehyde and incubated with rhodamine-wheat germ agglutinin (WGA). The top panels are phase contrast images; the bottom panels are rhodamine fluorescence.

(Figure 4A). Although the amount of G protein present in the enucleated cells was reduced, the rate of acquisition of endoglycosidase H resistance was found to be the same in the enucleated and control monolayers (Figure 4B).

Enucleated CHO cells have been previously shown to lack only the nucleus, the nuclear envelope, and a small amount of cytoplasm (approximately 10%) (22,23). The processes of protein synthesis, endocytosis, membrane ruffling and cell movement have been previously shown to occur in such enucleate cells (21,26). The current finding that the rate of transport of a viral glycoprotein from the RER to the Golgi apparatus is unaltered in enucleate cells strongly suggests that there is no obligatory role for the nuclear envelope in this intracellular transport step. Many questions remain regarding the role of the nuclear envelope in the membrane and secretory biosynthetic pathway. Perhaps the key question is whether the nuclear envelope translates and imports a different set of polypeptides than the bulk of the RER. The present data suggest that normally both the RER and NE can function in G protein assembly.

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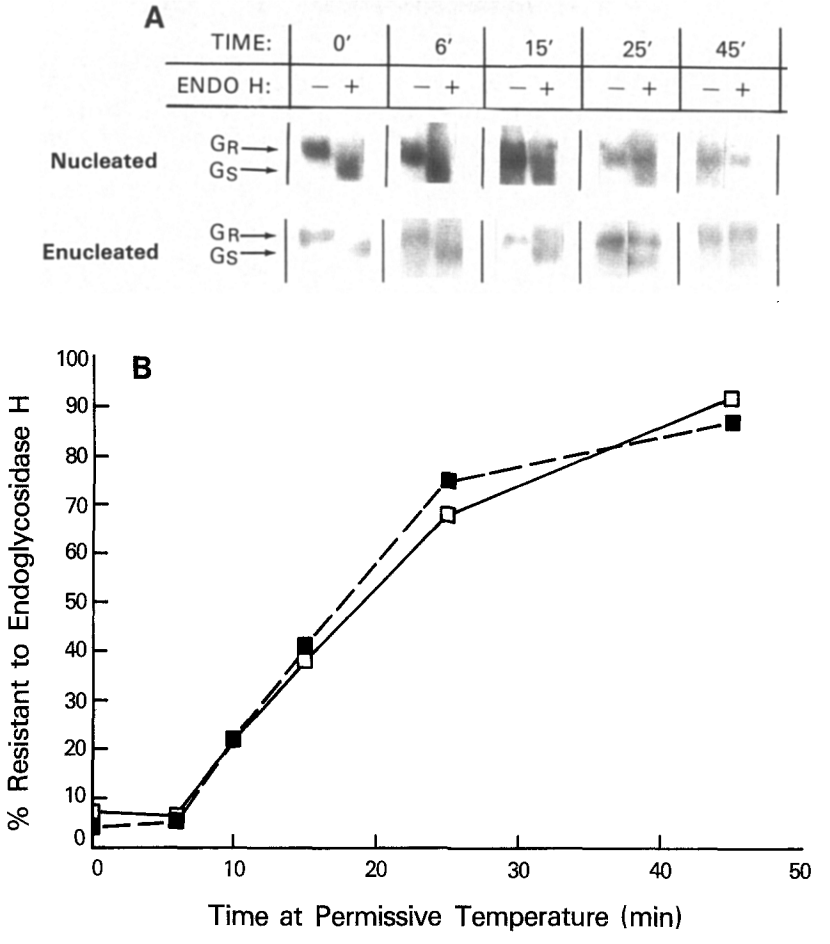

Figure 4. Temperature Sensitive transport of VSV ts O-45 G protein in Nucleated and Cytoplasts as assessed by Endoglycosidase H sensitivity.

CHO cells were infected at 37° C for 1 hour with VSV ts O-45. The cells were treated with cytochalasin B, or with control media. Following enucleation, the monolayers were labelled with ^{35}S-methionine for 3 hours at 40C. Synchronous transport to the Golgi apparatus was initiated by lowering the temperature to 32° C. At the indicated time points, G protein was extracted and immunoadsorbed using anti-G and N/NS proteins antisera (or anti-IKL antisera in other similar experiments). The immunoadsorbed material was either treated with Endo H or buffer alone for 12 hours at 37° C. The G protein was then analyzed by SDS-PAGE (Panel A) and the altered mobility of the G protein was detected by autoradiography and densitometry. These data were used to assess the kinetics of acquisition of resistance to endoglycosidase H (Panel B).

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