Identification and Characterization of a Mouse Cell Mutant Defective in the Intracellular Transport of Glycoproteins

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Abstract. We have isolated a mutant line of mouse L cells, termed gro29, in which the growth of herpes simplex virus (HSV) and vesicular stomatitis virus (VSV) is defective. The block occurs late in the infectious cycle of both viruses. We demonstrate that HSV and VSV enter gro29 cells normally, negotiate the early stages of infection, yet are impaired at a late stage of virus maturation. During VSV infection of the mutant cell line, intracellular transport of its glycoprotein (G protein) is slowed. Pulse-chase experiments showed that oligosaccharide processing is impeded, and immunofluorescence localization revealed an accumulation of G protein in a juxtanuclear region that contains the Golgi complex. We conclude that export of newly made glycoproteins is defective in gro29 cells, and speculate that this defect may reflect a lesion in the glycoprotein transport apparatus.

EUKARYOTIC cells utilize an elaborate transport system to move newly synthesized membrane and secretory proteins from their site of synthesis to their sites of action. Proteins synthesized in the endoplasmic reticulum (ER)[1] move to the Golgi complex before being sorted for transport to cellular organelles or the cell surface. Transport and sorting require an apparatus that ensures the delivery of individual proteins to correct locations. Although substantial progress is being made in identifying the protein determinants that influence transport and processing (reviewed by Garoff, 1985), less is known about how these determinants are recognized and acted upon by the cellular transport machinery.

One means of dissecting the transport apparatus of eukaryotic cells is to isolate cellular mutants defective in this process. This approach has begun to define the secretory pathway in yeast. Shekman and colleagues have isolated a large collection of mutants that are conditionally defective in particular steps in intracellular protein transport (Novick and Shekman, 1979; Novick et al., 1980; Esmon et al., 1981; Novick et al., 1981; Stevens et al., 1982). Genetic analyses of these secretion (*sec*) mutants indicate that the function of at least 20 different gene products is required between the time secretory proteins enter the ER and the time they are released at the plasma membrane.

Significant progress has also been made in the reconstitution of systems that carry out intracellular transport in vitro;

particularly from the study of cultured mammalian cells. Rothman and co-workers have documented the transfer of glycoproteins between isolated Golgi vesicles (reviewed by Dunphy and Rothman, 1985), and have used this reconstituted system to study vesicular transport in vitro. More recently, in vitro systems that carry out intravesicular transfer via the endocytic pathway have been described (Davey et al., 1985). Somatic cell mutants defective in glycoprotein processing (Gottlieb et al., 1975) have facilitated these in vitro studies. Unfortunately, attempts to isolate mammalian cell mutants defective in the transport process itself have met with little success. Selections for cells that are resistant to lectins, or cells that fail to express specific glycoproteins, have tended to yield mutants that are directly impaired in oligosaccharide synthesis or processing (reviewed by Stanley, 1984). To date, there are no reliable methods that facilitate the selection of animal cell mutants affected in protein transport.

During the course of a broader study to identify host cell functions required for herpes simplex virus (HSV) infection, we isolated a mouse L cell mutant that is defective in glycoprotein transport. This mutant, termed gro29, was isolated from a collection of mutagenized L cells that were selected for their ability to survive infection by HSV (Tufaro, F., and S. McKnight, manuscript in preparation). Initial experiments suggested that the block to HSV infection in this cell line occurred at a time when viral glycoprotein synthesis and virion assembly take place. The cellular defect also affected the production of vesicular stomatitis virus (VSV), which encodes a single envelope glycoprotein (G protein) that is essential for its infectivity.

In this paper we show that gro29 cells are defective in the transport of VSV G protein through the secretory apparatus to the cell surface. We also show by several criteria that endocytosis is unaffected in gro29 cells. It appears, therefore,

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1. *Abbreviations used in this paper*: endo H, endoglycosidase H; ER, endoplasmic reticulum; hpi, hours postinfection; HSV, herpes simplex virus; MOI, multiplicity of infection; pfu, plaque-forming unit; WGA, wheat germ agglutinin.

that the defect of these cells is specific to the exocytic transport pathway. To our knowledge this study represents the first characterization of a mammalian cell mutant that exhibits this phenotype.

Materials and Methods

Materials

The parental cells used for all experiments was the clone 1D line of LMtk⁻ mouse fibroblasts. HSV type 1 (KOS strain) was a gift from D. Coen (Harvard Medical School, Boston, MA). VSV (Indiana strain) was a gift from H. Lodish (Whitehead Institute for Biomedical Research, Cambridge, MA). Monoclonal antibody to VSV G protein was provided by L. Lefrancois (Upjohn Co., Kalamazoo, MI). Polyclonal antiserum against HSV-1 proteins was provided by G. Hayward (The Johns Hopkins University, Baltimore, MD). Fluorescein- and rhodamine-conjugated second antibodies were purchased from Cooper Biomedical, Inc., Malvern, PA.

Isolation of Mutant Cell Lines

Mouse L cells were grown in 150-mm plastic dishes containing Dulbecco's modified Eagle's medium and 10% fetal calf serum (DME-FCS). Roughly 1 × 10⁸ cells growing in three dishes were mutagenized for 18 h with 300 μg/ml ethyl methanesulfonate (Sigma Chemical Co., St. Louis, MO). After mutagenesis, the cells were grown for 2 d in DME-FCS, split 1:2, and grown for an additional 5 d before selection. Cells were infected with HSV-1 at an MOI (multiplicity of infection) of 1 plaque-forming unit (pfu)/cell. This resulted in the rapid death (within 72 h) of most mutagenized cells and all nonmutagenized (control) L cells. About 1 in 10⁶ of the original population of mutagenized cells survived to form colonies.

Indirect Immunofluorescence

Cells were grown on glass coverslips in the bottom of 35-mm plastic dishes. All further manipulations were performed at 20°C. For fixation, cells were rinsed with phosphate-buffered saline (PBS), and incubated in 1% formaldehyde in PBS for 10 min. Fixation was stopped by rinsing cells in PBS containing 1% bovine serum albumin (PBS-BSA). Cells were incubated in PBS-BSA containing 0.25% saponin (to permeabilize the cells) and appropriately diluted antiserum for 30 min, rinsed, and incubated in rhodamine-conjugated second antibody (goat anti-rabbit IgG for HSV antigen detection and goat anti-mouse IgG for VSV G protein detection). Cells were examined and photographed with a Zeiss IM-35 epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) using oil-immersion objectives and barrier filters.

For colocalization studies, cells were fixed for 15 min and washed in PBS-BSA for 20 min. To detect intracellular wheat germ agglutinin (WGA)-binding sites, cells were incubated for 90 min with 75 μg/ml unlabeled WGA, rinsed with PBS-BSA, and fixed for 15 min. Cells were then permeabilized with acetone, and incubated for 30 min with fluorescein-conjugated WGA (25 μg/ml in PBS supplemented with 700 μM calcium and 300 μM magnesium) to detect internal WGA-binding sites. VSV G protein was visualized in the same cells using anti-VSV G protein monoclonal antibody and rhodamine-conjugated goat anti-mouse IgG. Fields of cells were examined and photographed using filter combinations that eliminated crossover between rhodamine and fluorescein fluorescence channels.

Analysis of HSV-1-infected Cell Polypeptides

Cells were grown in 60-mm culture dishes and infected with HSV-1 at an MOI of 5 pfu/cell. At selected intervals (hours postinfection, hpi) cells were rinsed in labeling medium (minimal essential medium [MEM] containing 10% of the normal amount of methionine, normal amounts of nonessential amino acids and 2% FCS), and labeled for 3 or 9 h in medium containing 500 μCi/ml [³⁵S]methionine (Amersham Corp., Arlington Heights, IL). At the end of the labeling period, cells were lysed in SDS sample buffer (0.0625 M Tris, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Gels were dried and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

Pulse-Chase Labeling of VSV-infected Cells

Cells were grown in 35-mm culture dishes and infected with VSV at an MOI

of 10 pfu/cell. Infected cells were labeled with [³⁵S]methionine in methionine-free labeling medium as described by Zilberstein et al. (1980). Medium contained 2 μg/ml actinomycin D throughout infection. At 4 hpi, cultures were rinsed three times with labeling medium and then pulse-labeled for 10 min in 0.2 ml of labeling medium containing 200 μCi/ml [³⁵S]methionine. The medium was then removed, and the cultures were rinsed and fed with DME-FCS. At selected intervals after labeling, the cultures were washed with PBS and lysed in 1% NP-40, 1% sodium deoxycholate, 10 mM Tris HCl, pH 7.4, 150 mM NaCl. Cold acetone (4 vol) was added and precipitates were collected by centrifugation (1,000 g, 10 min). Virions were collected from the medium by centrifugation as described previously (Lodish and Kong, 1983). Pelleted samples were dissolved in 100 μl of 1% SDS, 5% β-mercaptoethanol, 0.1 M sodium citrate, pH 5.5. Aliquots (50 μl) were mixed with an equal volume of H₂O and incubated with endoglycosidase H (endo H, 80 ng, Boehringer Mannheim Biochemicals, Indianapolis, IN) for 18 h at 37°C. Endo H reactions were stopped by the addition of 0.5 vol of 3× SDS gel sample buffer. Samples were heated for 5 min at 95°C and electrophoresed on 10% polyacrylamide gels.

Pulse-Chase Labeling of Uninfected Cells

Cells growing in 100-mm dishes were pulse-labeled for 15 min with 0.5 mCi [³⁵S]methionine/dish in methionine-free labeling medium. The labeling medium was removed and the cells were washed two times with DME and fed 3 ml of DME. At appropriate intervals after labeling, the medium was removed and centrifuged to remove cell debris. The supernatant was supplemented with 10 μg of sodium deoxycholate and secreted proteins were precipitated by the addition of 4 vol of cold acetone. Cell-associated proteins were isolated as described for VSV-infected cells. Secreted and cell-associated proteins were solubilized in sample buffer, heated to 95°C, and size-separated on 6–12% polyacrylamide gels.

Radioiodination Assay of Cell Surface G Protein

Cells were grown in 35-mm culture dishes and infected with VSV at an MOI of 10 pfu/cell. At 4–5 hpi, the cells were labeled with ¹²⁵I by lactoperoxidase-catalyzed iodination at 4°C and lysed as described previously (Snider and Rogers, 1985). Lysates were incubated with anti-G protein monoclonal antibody for 2 h, goat anti-mouse IgG for 20 min, and protein A-Sepharose CL-4B for 2 h. All incubations were carried out at 4°C. The Sepharose beads were then washed extensively, as described previously (Snider and Rogers, 1985). Immunoprecipitates were dissolved in SDS sample buffer, heated to 95°C for 5 min and analyzed by electrophoresis on 10% polyacrylamide gels.

Binding and Internalization of Transferrin

The preparation of ¹²⁵I-human transferrin was carried out as described previously (Snider and Rogers, 1986). Parental and mutant cells growing in 35-mm dishes were washed three times with cold Hepes-buffered MEM containing 1 mg/ml BSA, and incubated for 30 min on ice with 3 μg/ml ¹²⁵I-transferrin. Cells were washed three times with cold Hepes-buffered MEM containing 1 mg/ml BSA and incubated at 37°C with Hepes-buffered MEM containing 60 μg/ml unlabeled transferrin and 1% fetal bovine serum. At various times post temperature-shift, cultures were chilled and washed with Hepes-buffered MEM containing 1 mg/ml BSA. Cell-associated radioactivity was determined by dissolving the cells in 0.5% sodium deoxycholate and 0.5 N NaOH and counting in a gamma counter. Internal radioactivity was measured by treating the cells briefly with acid prior to washing, which removes surface ligand (Klausner et al., 1983). Cell-surface radioactivity was calculated as the difference between the internal and total cell-associated radioactivity. Acid-insoluble radioactivity released into the medium was also measured. Values were corrected for nonspecific binding, which was measured in the presence of a 100-fold excess of unlabeled transferrin.

Results

In an attempt to identify host cell functions that are essential for HSV infection we employed a genetic strategy designed originally to study interactions between bacteriophage lambda and *Escherichia coli* (reviewed by Friedman et al., 1984). Host mouse L cells were mutagenized with ethyl methanesulfonate (EMS) and then exposed to HSV (see Materials

and Methods). Because the infection of cultured cells by HSV is highly cytotoxic, there is strong selection for host cell mutants that fail to propagate HSV. Using this method we selected 85 HSV-resistant clones from a starting population of roughly 10^8 L cells. Following the nomenclature adopted by Friedman, Herskowitz, and Georgopoulos, we term these *gro* mutants in accordance with their inability to support viral growth.

One mutant, designated gro29, failed to produce any infectious virus when challenged with HSV. This failure could re-sult from a block to initial penetration of the virus. Alternatively, HSV might enter gro29 cells normally, but be blocked at some step during the expression or assembly of viral gene products. In order to distinguish between these alternatives, we compared the expression of viral antigens in gro29 and parental L cells at successive intervals postinfection by indirect immunofluorescence using anti–HSV antiserum (Fig. 1). At 4 hpi, HSV antigens are observed in both cell types. The nuclei of infected cells exhibit the brightest fluorescence, consistent with the expression of immediate-early and
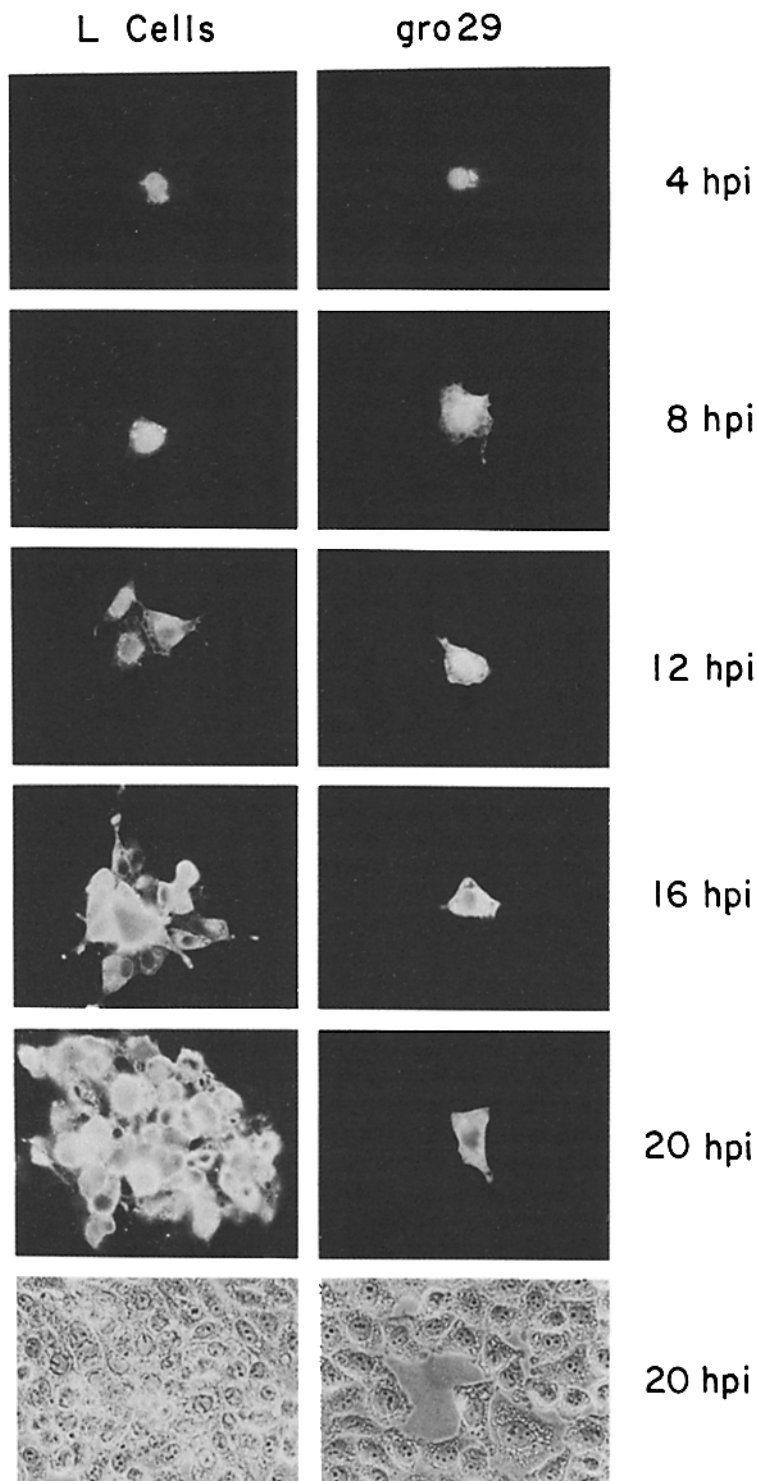
L Cells   gro29

4 hpi

8 hpi

12 hpi

16 hpi

20 hpi

20 hpi

Figure 1. Immunofluorescence analysis of HSV infection of mouse L cells and gro29 cells. Confluent monolayers of each cell type were inoculated with a diluted stock of HSV-1 such that roughly 1 cell in 10^4 became infected. The progress of the viral infection was assayed by indirect immunofluorescence using a polyclonal antiserum directed against HSV polypeptides (see Materials and Methods). Fluorescence micrographs of parental cells (*left*) and mutant cells (*right*) are shown. The values to the right indicate the hpi when the cells in each row were fixed for analysis. The bottom row shows phase-contrast micrographs of the same cell monolayers depicted in the 20-hpi timepoint.
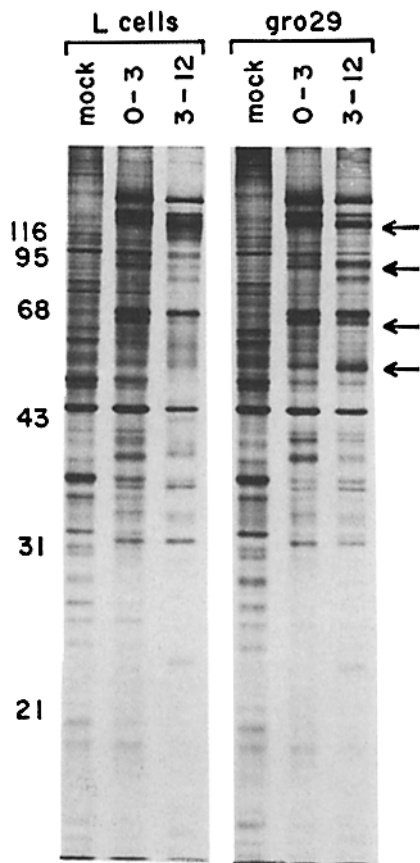
Figure 2. Comparison of the pattern of polypeptides synthesized in HSV-infected mouse L cells and gro29 cells. Monolayers of each cell type were infected at an MOI of 5 pfu/cell with HSV-1 and labeled with [^{35}S]methionine. Cells were solubilized and labeled protein analyzed by SDS-PAGE. The patterns of polypeptide synthesis in mock-infected cells labeled for 3 h (*mock*), and infected cells labeled 0–3 hpi (*0–3*) are the same for parental L cells and gro29 cells. The patterns differ, however, when the labeling period was carried out between 3 and 12 hpi (*3–12*). (*Arrows*) Polypeptides that accumulate to anomalously high levels in gro29 cells. Their apparent molecular masses are 51,000, 65,000, 90,000 and 110,000 daltons. Published estimates of the apparent molecular masses of the unprocessed HSV-1 glycoproteins are: gD = 51,000, gE = 66,000, gC = 86,000–105,000, gA/B = 110,000 daltons (reviewed by Campadelli-Fiume and Serafini-Cessi, 1985).

delayed-early viral proteins which are known to localize within the nucleus. At 8 hpi, fluorescence is again observed in both parental and mutant cells. By this time, however, the fluorescence is dispersed throughout both nucleus and cytoplasm. In that viral antigens appear to accumulate in gro29 cells with the same kinetics as in parental L cells, and in that the same number of cells become infected when inoculated with a constant amount of virus (data not shown), we conclude that HSV enters gro29 cells and begins its infectious cycle normally.

Release of infectious virus by parental L cells is first detected at 12 hpi. This is clearly demonstrated in Fig. 1 where it can be seen that viral infection has spread to neighboring cells. Parental L cells continue to release virus, and by 20 hpi as many as 80 cells are included within each infectious center. In contrast, gro29 cells show no spread of the initial

infection. Thus, despite the fact that gro29 cells synthesize viral antigens (Fig. 1) and viral DNA (data not shown) in an unimpeded manner, they appear incapable of releasing infectious virus.

To follow the course of HSV infection of gro29 cells more closely, we radiolabeled proteins at several intervals postinfection and fractionated them on SDS polyacrylamide gels (Fig. 2). Uninfected gro29 cells synthesized an array of polypeptides that was indistinguishable from parental L cells. When each cell type was infected with HSV and radiolabeled 0–3 hpi, the patterns of protein synthesis were again indistinguishable. That is, HSV succeeded in extinguishing host protein synthesis and inducing its own program of early gene expression in both cell types. In contrast, when the radiolabeling period was carried out between 3 and 12 hpi, HSV-infected gro29 cells did show differences from parental L cells. Four abundant polypeptide species were observed in gro29 cells that were not detected in L cells (Fig. 2, *arrows*). These observations suggest that a class of late viral proteins is aberrantly induced or processed in gro29 cells.

The four polypeptides that accumulate aberrantly in gro29 cells exhibit electrophoretic mobilities similar to immature HSV glycoproteins (Johnson and Spear, 1982; Campadelli-Fiume and Serafini-Cessi, 1985). Because it is known that pharmacologic blocks to glycoprotein transport or processing can inhibit HSV propagation (Johnson and Spear, 1982; Serafini-Cessi et al., 1983), we reasoned that gro29 cells might be defective in the processing or transport of membrane glycoproteins. To evaluate this hypothesis, we examined the ability of gro29 cells to synthesize, process, and transport the envelope glycoprotein (G protein) of VSV. We chose VSV because the processing and transport of G protein has been thoroughly studied. As a first step, we performed virus plaque assays to compare VSV growth in L cells and gro29 cells. VSV formed the same number of plaques on each cell type, indicating that binding and uptake of virus occurs normally in gro29 cells (data not shown). The plaques formed on gro29 cells, however, were substantially smaller than those formed on L cells. These observations indicate that the block to VSV propagation in gro29 cells occurs after entry, and are consistent with the possibility that gro29 might impede the processing or transport of VSV G protein.

We next monitored oligosaccharide processing and transport of G protein directly. In normal cells, G protein is synthesized on membrane-bound polyribosomes in the rough ER, and then transported through the Golgi complex to the cell surface. Two asparagine-linked oligosaccharides are added to the polypeptide as high-mannose precursors which are converted to complex-type structures in the Golgi complex. Because endo H cleaves high-mannose chains, but not complex oligosaccharides, it serves as a useful probe for the processing of VSV G protein.

Parental and gro29 cells were infected with VSV, pulse-labeled with [^{35}S]methionine for 10 min, and chased for various times up to 3 h. Cells were lysed and a fraction of each lysate was digested with endo H. Digested and undigested aliquots were then analyzed by SDS-PAGE. We observed that all of the polypeptides encoded by VSV (L, G, N, NS, and M) were synthesized in comparable amounts in pulse-labeled L cells and gro29 cells (Fig. 3). Moreover, the G protein that was synthesized in the two cell lines was of similar size and was sensitive to digestion by endo H. Appar-
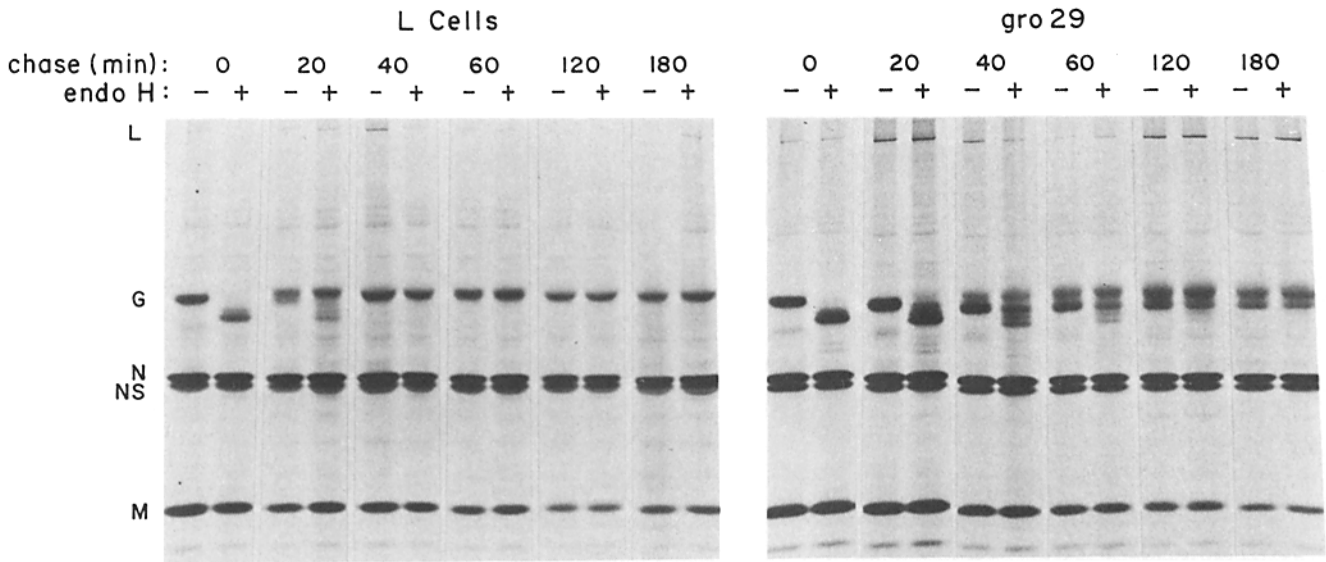
Figure 3. Assays of synthesis and processing of VSV proteins in mouse L cells and gro29 cells. Monolayers of each cell type were infected at an MOI of 10 pfu/cell with VSV in the presence of 2 µg/ml actinomycin D. Cells were pulse-labeled for 10 min with [^{35}S]methionine and chased for the indicated times. Aliquots of cell lysates were digested with endo H (see Materials and Methods). Digested (+) and mock-digested (−) lysates were subjected to SDS-PAGE. The five VSV proteins are identified by letters at the left of the figure.

ently, insertion into the rough ER, cleavage of the signal peptide, and addition of high-mannose oligosaccharides to VSV G protein all occur normally in gro29 cells. The conclusion that early steps in the synthesis and posttranslational processing of G protein occur normally in gro29 cells is further supported by analyses of the oligosaccharides added to G protein in cells pulse-labeled with [^{3}H]mannose. In pulse-labeled parental and gro29 cells, GlcMan$_9$GlcNAc$_2$ is the predominant G protein oligosaccharide (M. Snider, unpublished). This is the oligosaccharide normally found attached to G protein shortly after its synthesis (Li et al., 1978).

Although the initial synthesis of VSV G protein appears to

occur normally in gro29 cells, subsequent processing of the high-mannose oligosaccharides is clearly aberrant (Fig. 3). In parental L cells, G protein becomes endo H-resistant very rapidly; processing is complete between 20 and 40 min after labeling. These observations are in agreement with previous reports that have demonstrated the rapid processing of VSV G protein (Strous and Lodish, 1980; Lodish and Kong, 1983; Rose and Bergmann, 1983; Machamer et al., 1985). In contrast to the rapid processing of G protein in L cells, gro29 cells are slow to convert the endo H-sensitive, high-mannose oligosaccharides of G protein to the endo H-resistant state. By 40 min after labeling, only 50% of the G protein has
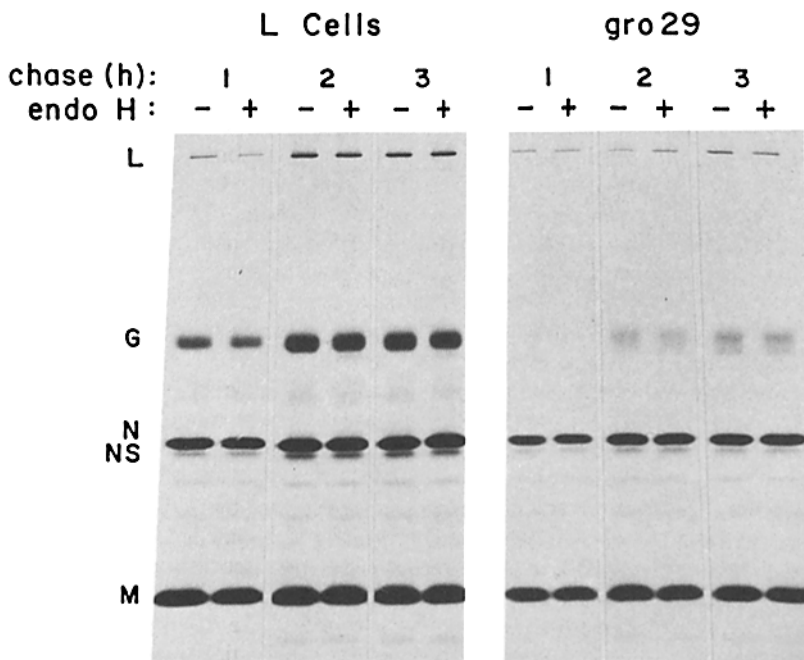

Figure 4. Comparison of the rate of appearance of newly synthesized VSV G protein in virions released from mouse L cells and gro29 cells. Virions were isolated from the chase medium in a pulse-chase experiment identical to the one shown in Fig. 3. Aliquots of each sample were digested with endo H. Digested (+) and mock-digested (−) samples were subjected to SDS-PAGE. The time after pulse-labeling when the virions were collected is indicated above each pair of lanes. The five VSV proteins are identified at the left of the figure.
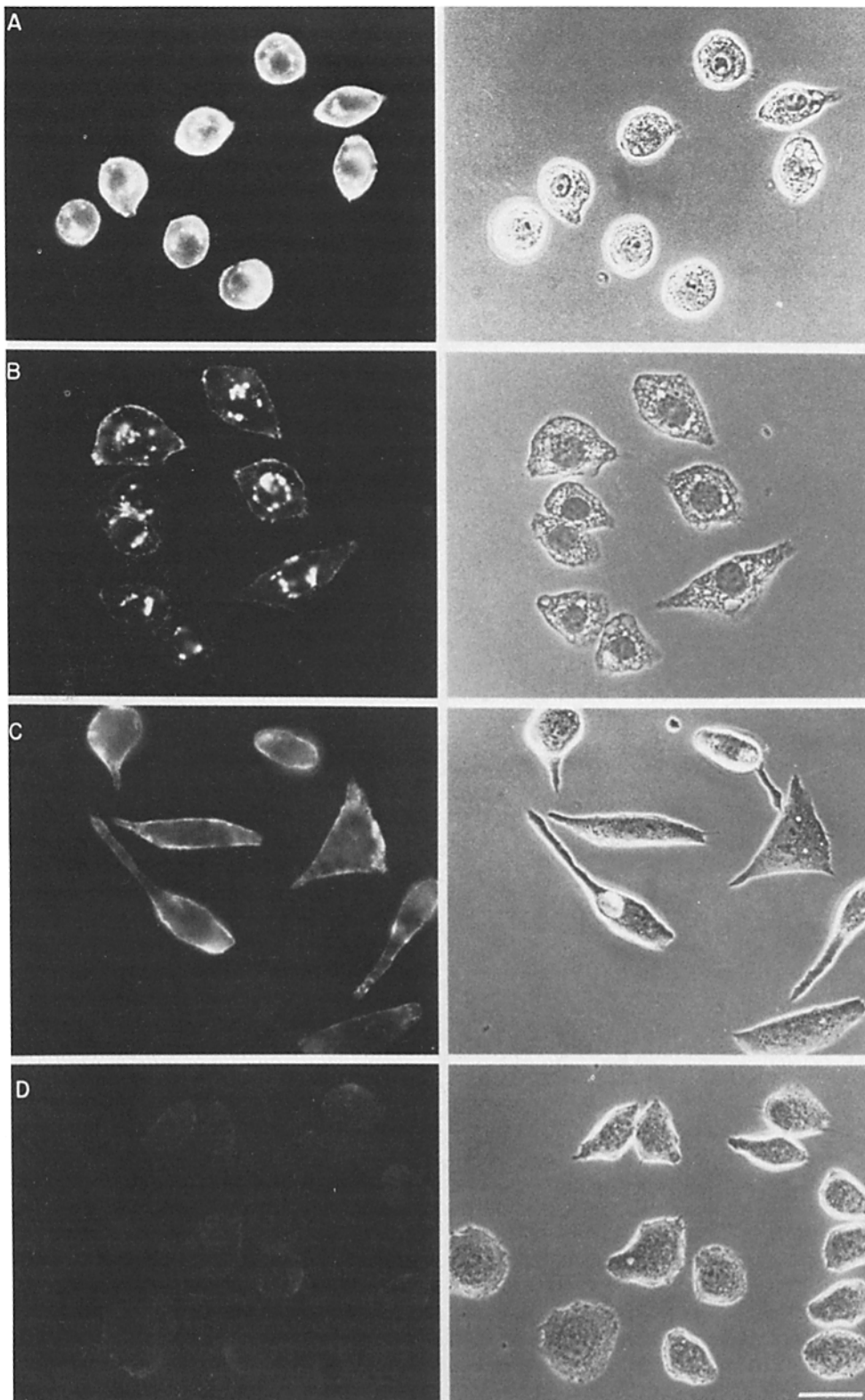
Figure 5. Localization of G protein in VSV-infected mouse L cells and gro29 cells. Cells were grown on glass coverslips and infected at an MOI of 5 pfu/cell with VSV. In order to detect total G protein, cells were fixed at 4 hpi, permeabilized, and probed with a monoclonal antibody specific to VSV G protein (see Materials and Methods). Fluorescence micrographs of (*A*) parental L cells and (*B*) gro29 cells are shown in the left column (corresponding phase-contrast micrographs of the same fields are shown in the right column). To visualize surface G protein, living cells were incubated at 4 hpi with anti–VSV G, followed by fluorescein-conjugated goat anti–mouse Fab. Fluorescence and phase-contrast micrographs of (*C*) parental L cells and (*D*) gro29 cells are shown. Bar, 40 μm.

achieved the endo H-resistant state. The data presented in Fig. 3 also show that gro29 cells accumulate novel forms of VSV G protein. One form appears to be smaller than mature G protein and another appears larger (diagnosed by relative electrophoretic mobilities). In gro29 cells 54% of radiolabeled G protein accumulates as the largest species (see Fig.

3). These novel forms presumably represent species of G protein that have abnormal oligosaccharides. The structures of these aberrant oligosaccharides are currently being examined. Coupled with the delayed acquisition of sensitivity to endo H, these data provide strong evidence that gro29 cells are defective in the processing of VSV G protein.

One explanation for the phenotype of gro29 cells is that they are defective in enzymatic functions directly required for the conversion of high-mannose oligosaccharides to their more mature forms Alternatively, the defect may impair the transport of glycoproteins from the ER through the Golgi complex to the cell surface. To determine whether transport is impeded in gro29 cells, we tracked the appearance of labeled VSV proteins in virions during the chase period of an experiment identical to that described in Fig. 3. Culture medium was collected 1, 2, or 3 h after the pulse. Virions were then isolated from the medium by centrifugation, solubilized, digested with endo H, and electrophoresed on an SDS-polyacrylamide gel (Fig. 4). When virions derived from gro29 cells were compared with those derived from parental L cells, similar amounts of all virion proteins were observed with one exception. After 1 h of chase, the amount of labeled G protein in virions, normalized to the amount of labeled N+NS proteins is only 4% the level of parental L cells. By 3 h after labeling, this value reached only 30%. We have not determined whether virions released from gro29 cells contain the same amount of G protein (labeled and unlabeled) as those released from L cells. However, the impeded flux of labeled G protein into virions released from gro29 cells supports the possibility that this mutant may be defective in the intracellular transport of glycoproteins.

Based on these results, we reasoned that VSV G protein might accumulate within infected gro29 cells. Likewise, the amount of G protein that reached the cell surface of gro29 cells might be expected to be reduced relative to parental L cells. Two experiments were performed to test these hypotheses. First, the location of VSV G protein in parental and mutant cells was studied by immunofluorescence microscopy using an anti-VSV G protein monoclonal antibody. In parental L cells that have been infected with VSV, fluorescence is observed on both surface and intracellular structures (Fig. 5 A). In contrast, VSV-infected gro29 cells exhibit intense juxtanuclear fluorescence and minimal surface fluorescence (Fig. 5 B). To visualize the cell surface-associated G protein exclusively, living cells were incubated with the anti-VSV G monoclonal antibody followed by a fluorescein-conjugated Fab fragment. As expected, VSV-infected parental L cells exhibit strong surface fluorescence (Fig. 5 C) when compared with the amount detected on the surface of gro29 cells (Fig. 5 D).

The second approach that was used to study G protein transport involved immunoprecipitation of surface-labeled proteins. After lactoperoxidase-catalyzed iodination, cell-surface proteins were solubilized and subjected to immuno-precipitation with a monoclonal antibody to VSV G protein. Precipitated protein was sized by SDS-PAGE, which allowed quantitation of the amount of G protein on the surface of parental and mutant cells. These data (not shown) indicate that at 5 hpi, gro29 cells contain only 25% as much G protein on their surface as parental L cells.

We attempted to define the intracellular compartment of gro29 cells where VSV G protein accumulates by comparing the G protein distribution to that of binding sites for WGA. WGA binds to terminal sialic and GlcNAc residues of glycoproteins which are added in the Golgi complex (reviewed by Schachter and Roseman, 1980); thus WGA-binding sites are found in the Golgi apparatus, as well as in organelles containing molecules that have passed through the Golgi com-

plex (Virtanen et al., 1980). To evaluate the location of VSV G protein and WGA in the same cells, we carried out double-labeling experiments using fluorescein-labeled WGA and rhodamine-labeled goat anti-mouse IgG. The upper panel of Fig. 6 shows the location of VSV G protein in gro29 cells, and the lower panel shows the distribution of WGA-binding sites in the same cells. The two markers exhibit substantial colocalization, particularly in a juxtanuclear structure that probably corresponds to the Golgi complex.

It is unlikely that the defect in gro29 is restricted to the transport of viral glycoproteins. Uninfected gro29 cells exhibit a doubling time twice that of parental L cells. They also fail to grow in reduced serum medium that is known to support the growth of parental L cells (Tufaro, F., unpublished observation). In the light of these observations, we extended our characterization of gro29 cells to determine whether the secretion of endogenous proteins was impeded relative to parental L cells. L cells and gro29 cells were pulse-labeled with [^{35}S]methionine. Cell-associated and secreted proteins were isolated at several intervals after labeling and analyzed by SDS-PAGE (Fig. 7). We observed no marked difference in the qualitative pattern of polypeptides synthesized by the two cell types, nor did we notice any quantitative difference in label incorporation. In contrast, we did observe distinct differences in the patterns of polypeptides secreted during the chase period. Of four prominent polypeptides secreted by both cell types (indicated by arrows), three were secreted from gro29 cells with delayed kinetics. Furthermore, a number of polypeptides appeared to migrate on SDS polyacrylamide gels as discrete species when secreted from parental L cells, but as diffuse species when secreted from gro29 cells.

Observations presented thus far suggest that gro29 cells may be defective in the transport of newly synthesized proteins to the cell surface. If this is the case, it is of interest to know whether endocytic processes are also affected in this mutant. This was tested by comparing the receptor-mediated endocytosis of transferrin in parental and gro29 cells. Transferrin, the serum iron carrier, binds to a specific receptor on the cell surface and is rapidly internalized. The iron then dissociates, most likely in the acidic endosomal compartment, and the receptor–transferrin complex returns to the cell surface where apoprotein-transferrin is released (reviewed by May and Cuatrecasas, 1985).

To examine the endocytosis and release of transferrin, parental and gro29 cells were exposed to ^{125}I-transferrin, washed and incubated in unlabeled culture medium at 37°C. At various times after binding, the amounts of surface and internal ^{125}I-transferrin were determined. In addition, ^{125}I-transferrin release into the culture medium was measured at each time point. Parental and gro29 cells contain similar numbers of cell surface transferrin receptors. As Fig. 8 shows, more importantly, they internalize and release ^{125}I-transferrin (^{125}I-Tf) into the medium at similar rates. In both cell types most of the surface ^{125}I-transferrin is internalized within 10 min, and 80% is returned to the surface and released into the medium within 30 min. These results suggest that endocytic processes occur normally in gro29 cells.

Discussion

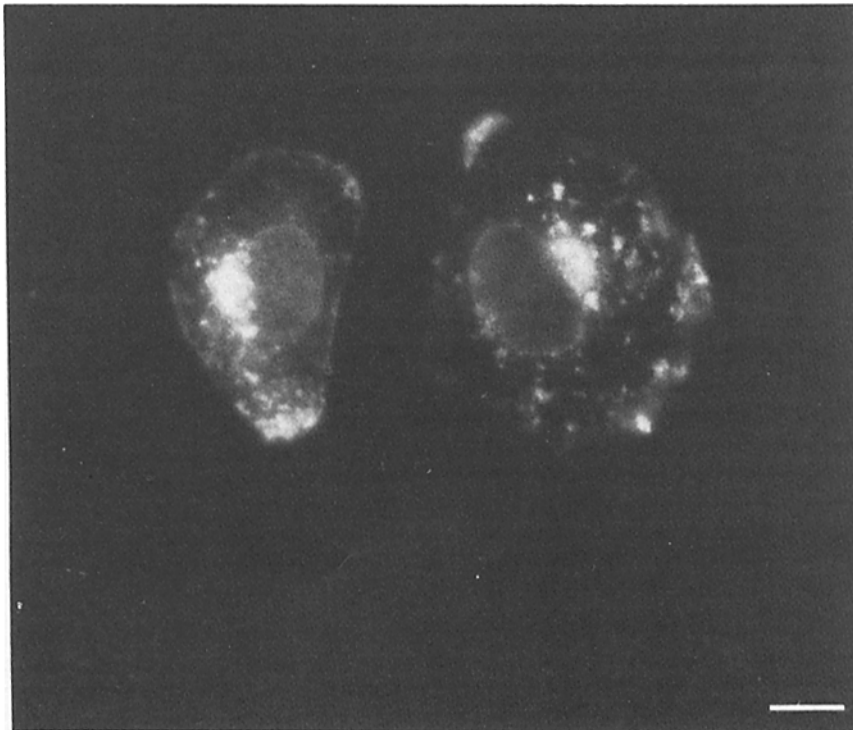This report describes the preliminary characterization of a

G protein
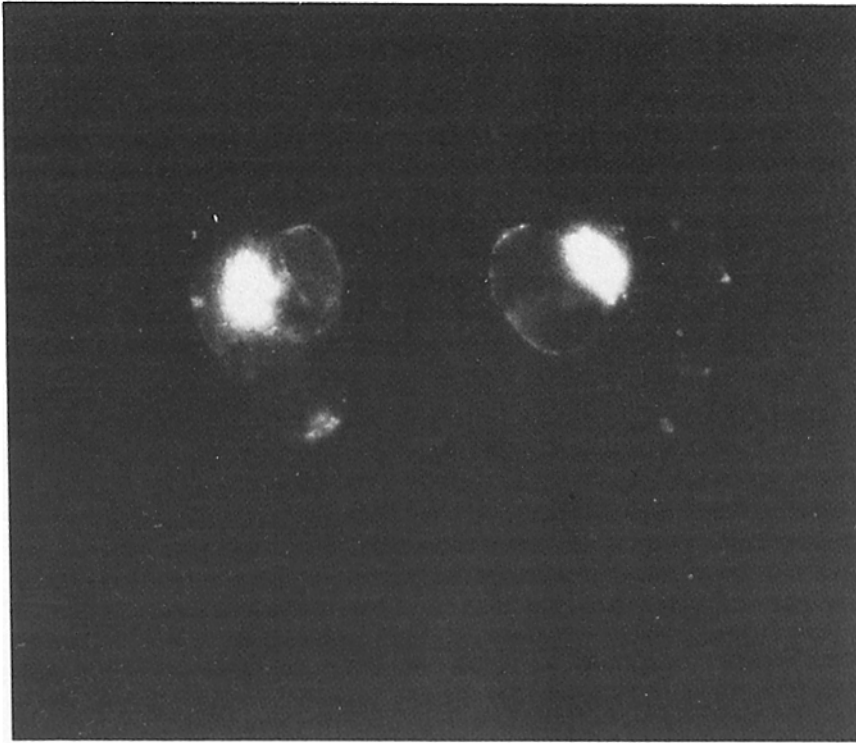
WGA

Figure 6. Localization of VSV G protein to a juxtanuclear compartment of gro29 cells. gro29 cells were grown on glass coverslips and infected at an MOI of 10 pfu/cell with VSV. Cells were fixed at 4 hpi, and Golgi and post-Golgi regions were visualized with fluorescein-conjugated wheat germ agglutinin (see Materials and Methods). VSV G protein was visualized in the same cells by indirect immunofluorescence using an anti-G monoclonal IgG followed by rhodamine-conjugated goat anti–mouse IgG. Fluorescence micrographs revealing G protein (*top*) and WGA (*bottom*) in the same field of cells were obtained using barrier filters that prevent crossover between rhodamine and fluorescein fluorescence. Bar, 15 μm.
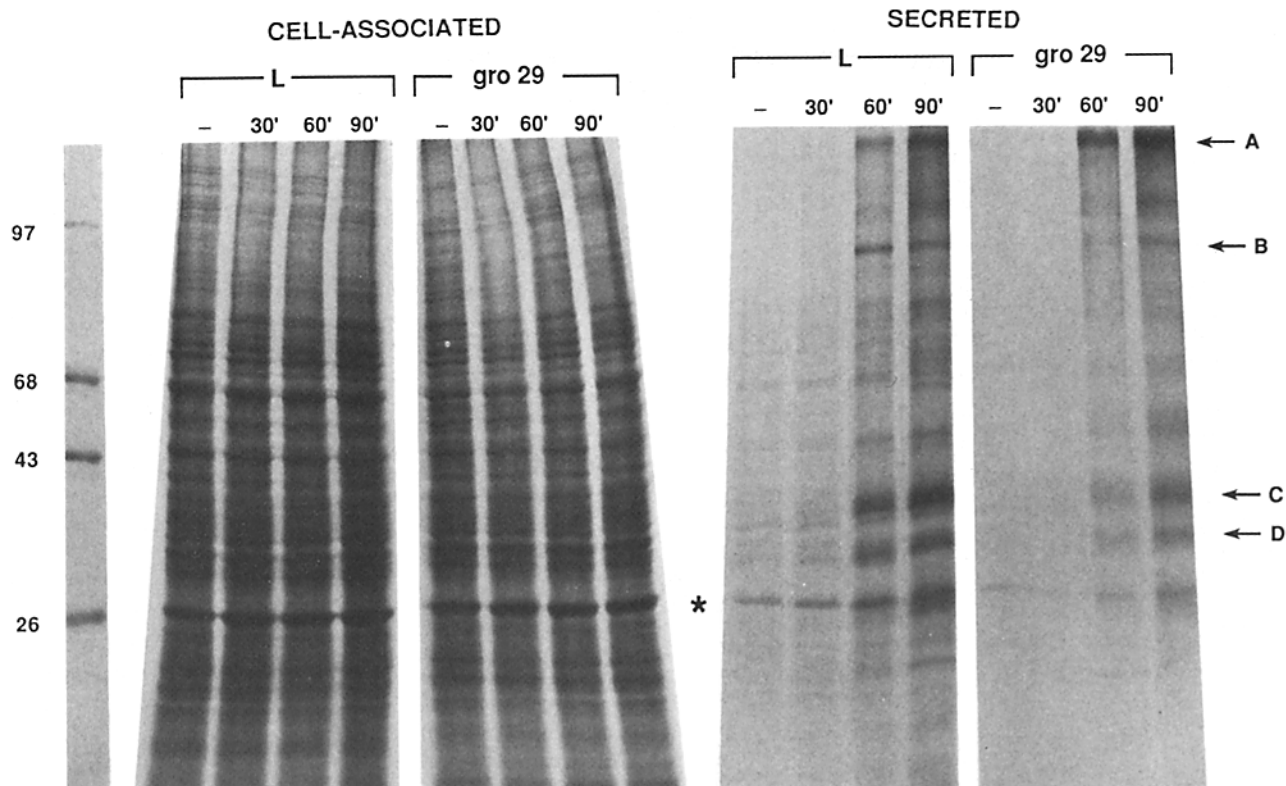
Figure 7. Comparison of newly synthesized polypeptides secreted from mouse L cells and gro29 cells. Monolayers of each cell type were pulse-labeled for 15 min with [^{35}S]methionine and chased for the indicated times. Cell-associated proteins were prepared by lysing cell monolayers after extensive washing with PBS (see Materials and Methods). Aliquots of cell-associated and secreted proteins were subjected to SDS-PAGE. (*Asterisk*) A prominent cell-associated protein that leaked into the secreted fraction. (*A, B, C,* and *D*) Prominent secreted polypeptides. Polypeptides *B, C,* and *D* are underrepresented in the culture supernatant from gro29 cells at the 60-min timepoint after labeling. Molecular mass markers (kD) are shown at the left of the figure.

mammalian cell mutant that is defective in the intracellular transport of glycoproteins. Although we have not as yet mapped or identified the mutation that causes gro29 cells to display the transport-defective phenotype, we believe that it is a bona fide somatic cell mutant. First, HSV-resistant mutants were retrieved from our initial screen at a frequency similar to that reported for the generation of other somatic cell mutants (about 1 in 10^6). Second, the phenotype of gro29 is stable; it reverts to the HSV-sensitive phenotype at a rate lower than 1 in 10^6 (Tufaro, F., and S. McKnight, unpublished data). Finally, the phenotype of gro29 appears to be unrelated to its initial exposure to HSV; it does not contain episomal or integrated copies of the HSV genome (Tufaro, F., and S. McKnight, unpublished observation), and it does not express viral antigens in the absence of infection (see Fig. 1).

How could a mutant blocked late in infection have survived our selection procedure? One possibility is that the surviving mutants escaped initial HSV infection during the selection. In that mutagenized cells were allowed to grow for 7 d before infection, small colonies of mutant cells were formed. When the cultures were subsequently inoculated with virus, cell colonies, particularly mutants incapable of virus propagation, might have provided a "microenvironment" that protected central cells from infection. Alternatively, gro29 cells might be capable of surviving the cytopathic effects induced by HSV infection prior to the egress of virions from the cell.

We are currently testing this possibility. Regardless of the reason for the survival of gro29, it is clear that this selection procedure is useful for isolating mutants that interfere with the HSV lytic infectious cycle.

We have shown by a number of different criteria that VSV G protein transport is defective in gro29 cells. These include biochemical demonstration of the retardation of oligosaccharide maturation on newly made VSV G protein, and immunologic demonstration of the accumulation of G protein in a juxtanuclear compartment of infected cells. Perhaps the most convincing demonstrations of the transport defect in gro29 cells come from measurements of G protein flux to the surface of infected cells, and from direct measurements of the rate of appearance of G protein in virions. In both cases we were able to document a marked lag in the intracellular transport of G protein.

What type of physiologic defect could account for the phenotype of gro29? For several reasons we doubt that its phenotype results from an alteration in an enzymatic step involved in oligosaccharide synthesis or processing. First, inhibitors of oligosaccharide processing, including deoxymannojirimycin and deoxynojirimycin, potent inhibitors of Golgi mannosidase I and ER glucosidases, respectively, drastically alter oligosaccharide processing but do not affect intracellular G protein transport (Burke et al., 1984; Schlesinger et al., 1984). Second, several different defects in the processing of VSV G protein oligosaccharides are seen in gro29 cells, in-
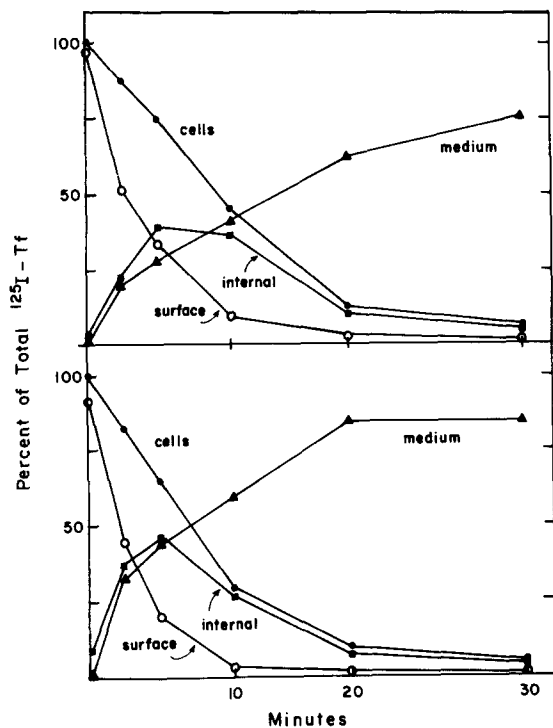
Figure 8. Endocytosis and release of transferrin are similar in mouse L cells and gro29 cells. Cells were incubated with ^{125}I-transferrin for 30 min at 0°C to saturate surface binding sites. They were then washed and incubated at 37°C in medium containing unlabeled transferrin for the indicated times. The amount of radioactive transferrin that was cell associated (●), surface associated (○), internal (■) or free in the medium (▲) was determined for L cells (*top panel*) and gro29 cells (*bottom panel*) as described in Materials and Methods.

cluding slow acquisition of endo H resistance, as well as the accumulation of novel mature forms. If these defects are due to a single lesion in the glycosylation pathway, then the lesion must occur very early in oligosaccharide synthesis. However, we have found that the early stages of asparagine-linked glycosylation are normal in gro29 cells; the synthesis of the precursor oligosaccharide, its transfer to protein, and the removal of glucose residues by ER glucosidases are identical in mutant and parental cells (Snider, M., unpublished observation).

An alternative explanation for the defect of gro29 cells is that it affects several stages of membrane glycoprotein transport including passage from the ER to the Golgi complex, and subsequent movement to the cell surface. A reduction in the rate of interorganelle transfer would account for the slow rate of oligosaccharide processing as well as for the slow transit of VSV G protein from ER to the cell surface. If such is the case, some element common to all of these transfer reactions might be defective, possibly related to the formation, movement or fusion of transport vesicles.

It may be worth noting that the defects of gro29 cells are similar to some of the effects of the monovalent cation ionophore monensin (Johnson and Schlesinger, 1980). Monensin disrupts the morphology of the Golgi apparatus, causing a distinct swelling of the cisternae (Quinn et al., 1983). Monensin is also known to retard transport of newly made membrane glycoproteins and secretory proteins (Tartakoff

and Vassalli, 1977, 1978; Johnson and Schlesinger, 1980; Strous and Lodish, 1980; Tartakoff, 1983). In the presence of monensin, glycoproteins are transported into the medial Golgi compartment where they are converted to endo H-resistant forms, but do not progress to the *trans* compartment. It is possible that the smaller endo H-resistant form of G protein observed in gro29 cells represents material that has been exposed to GlcNAc transferases in the medial Golgi (Dunphy et al., 1985), but has not been acted on by the terminal Gal and sialytransferases in the *trans* compartment of the Golgi complex. In addition, if G protein is kept in a Golgi compartment for an abnormally long time, oligosaccharide processing activities specific to that compartment might process G protein aberrantly. For example, the larger G protein species may represent oligosaccharides that are "oversialylated" due to an extended lifespan in the *trans* compartment of the Golgi complex. Direct tests of these hypotheses can now be carried out by determining the structures of the oligosaccharides that are found on VSV G protein in gro29 cells.

It is unlikely that gro29 is defective in all aspects of vesicular transport. We found that the receptor-mediated uptake and release of transferrin occurs normally in the mutant cell line. Moreover, VSV, which enters cells by endocytosis (Marsh, 1984), penetrates gro29 cells in an unimpeded manner. Finally, gro29 and parental L cells are equally sensitive to the cytotoxic lectin modeccin (Tufaro, F., unpublished observation). Because modeccin enters cells by endocytosis (Sandvig and Olsnes, 1982), this result also suggests that endocytosis is normal in gro29 cells.

We are aware of two reports in the literature that concern variant mammalian cells affected in intracellular protein trafficking. Robbins et al. (1984) have isolated a Chinese hamster ovary (CHO) cell mutant, termed DTG 1-5-4, that is defective in receptor-mediated endocytosis and partially impaired in glycoprotein maturation. Although the latter phenotypic property of DTG 1-5-4 is similar to that ascribed to gro29 in this report, it is improbable that they have suffered lesions in the same genetic locus. Unlike gro29, DTG 1-5-4 cells are highly resistant to modeccin and defective in endocytosis. Nakano et al. (1985) have described another CHO mutant, termed DS28-6, that is conditionally defective in protein export. DS28-6 is reported to be incapable of releasing VSV virions at its nonpermissive temperature. Informative comparisons among gro29, DTG 1-5-4, and DS28-6 are problematic in that the mutants have been characterized either by different functional tests or to different levels of detail.

Perhaps the most surprising observation to come from this study is the complete inability of infected gro29 cells to shed infectious HSV. In contrast, gro29 cells produce ~35% of the normal amount of infectious VSV particles. It is perhaps the case that the relevant difference in the production of these two viruses by gro29 cells is due to the different sites of virion assembly. VSV assembly begins in the cytoplasm and is completed when nucleocapsids bud from the plasma membrane. In contrast, HSV virions begin assembly in the nucleus, become enveloped as they exit the nucleus, and are then believed to be transported from the ER to the Golgi complex en route to the plasma membrane (Johnson and Spear, 1982). We speculate that the egress of HSV virions taxes the intracellular transport apparatus to its limits. Thus, defects that are sublethal to the transport of cellular glyco-

proteins (as well as VSV G protein), might completely eliminate the production of infectious HSV.

Regardless of the explanation for the effect of the lesion in gro29 cells on the release of infectious HSV virions, we have shown that the defect is extremely strong. We are encouraged that the selection scheme outlined in this report will facilitate the isolation of additional mutants that are defective in glycoprotein transport. Indeed, preliminary studies indicate that more than half of the HSV-resistant lines that we have isolated block virus propagation at a late stage of the infectious cycle (Tufaro, F., and S. McKnight, unpublished observation). We hope that this study represents the starting point for a genetic attack on glycoprotein transport in mammalian cells.

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