A Single Mutation in Chinese Hamster Ovary Cells Impairs Both Golgi and Endosomal Functions

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ABSTRACT A Chinese hamster ovary cell mutant DTG 1-5-4, was selected for pleiotropic defects in receptor-mediated endocytosis by methods previously described (Robbins, A. R., S. S. Peng, and J. L. Marshall, 1983, *J. Cell Biol.*, 96:1064–1071). DTG 1-5-4 exhibited increased resistance to modeccin, Pseudomonas toxin, diphtheria toxin, Sindbis virus, and vesicular stomatitis virus, as well as decreased uptake via the mannose 6-phosphate receptor. Fluorescein–dextran-labeled endosomes isolated from DTG 1-5-4 were deficient in ATP-dependent acidification in vitro. Endocytosis and endosome acidification were both restored in revertants of DTG 1-5-4 and in hybrids of DTG 1-5-4 with DTF 1-5-1, another endocytosis mutant exhibiting decreased ATP-dependent endosome acidification.

Both DTG 1-5-4 and DTF 1-5-1 were blocked at two stages of infection with Sindbis virus: at low multiplicities of infecting virus, resistance reflected a block in viral penetration into the cytoplasm, but at higher multiplicities of infection the block was in virus release. Like endocytosis, release of Sindbis virus was increased in revertants of DTG 1-5-4 and in DTG 1-5-4 × DTF 1-5-1 hybrids. Decreased release of virus from DTG 1-5-4 correlated with defects in some of the Golgi apparatus-associated steps of Sindbis glycoprotein maturation: proteolytic processing of the precursor pE2, galactosylation, and transport to the cell surface all were inhibited. In contrast, mannosylation, fucosylation, and acylation of the Sindbis glycoproteins, and galactosylation of vesicular stomatitis virus and cellular glycoproteins occurred to similar respective extents in mutant and parent. Electron microscopic examination of Sindbis-infected DTG 1-5-4 showed a remarkable accumulation of nucleocapsids bound to cisternae adjacent to the Golgi apparatus; virions were observed in the lumina of some of these cisternae.

That the alterations in both endocytosis and Golgi-associated steps of viral maturation result from a single genetic lesion indicates that these processes are dependent on a common biochemical mechanism. We suggest that endocytic and secretory pathways may share a common component involved in ion transport.

Acidification of intracellular organelles is essential for many late events in receptor-mediated endocytosis (reviewed in references 1 and 2). In those systems in which receptors recycle, re-use of receptors may require the dissociation of bound ligand at pH <6.0 (3–5). The transferrin system is an interesting variation on this theme, in that at acidic pH the ligand is not dissociated from the receptor, but instead releases its bound iron (6, 7). Some toxins and enveloped viruses depend on an acidic environment for penetration into the cytosol (8–11). In addition, lysosomal degradation of endocytosed ligand occurs at acidic pH.

Lysosomes were initially thought to be the only acidic organelles in the endocytic pathway; recently, acidic prelysosomal organelles, the endosomes, have been reported (12, 13).

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Endosomes comprise a heterogeneous population of vesicles and tubules that serve as intermediates in the transport of internalized macromolecules to lysosomes (1). Dissociation of asialoglycoprotein–receptor complexes in hepatocytes (14), release of iron from transferrin (15), and penetration of Semliki Forest virus into the cytosol of baby hamster kidney-21 cells (16) have been shown to occur in endosomes. Like lysosomes (17, 18), endosomes lower their internal pH via an ATP-driven proton pump (13). Lysosomal (19) and endosomal (13) proton-ATPases, as well as proton-ATPases of liver Golgi fractions (20), brain-coated vesicles (21), and chromaffin granules (22) exhibit similar properties; thus, these various pumps may be related.

Agents that dissipate transmembrane pH gradients, e.g., NH_4Cl, chloroquine, and monensin, have been used to demonstrate the importance of acidification in endocytosis (4, 5, 8–11, 14, 23). Inasmuch as these drugs affect all acidic compartments within the cell, their value in defining individual steps in the endocytic pathway is limited. As an alternative approach, several laboratories have isolated mutant cell lines with pleiotropic defects in receptor-mediated endocytosis (24–26). Merion et al. have shown that two such mutants are defective in the ATP-dependent acidification of endosomes in vitro (27). Klausner et al. (28) have presented evidence indicating a similar defect in a third mutant. A primary defect in endosome acidification appeared to explain the various alterations in endocytosis observed in these mutants. However, one observation indicated that the problem might extend beyond the endocytic pathway—plaques formed by Sindbis virus on monolayers of the mutants were always smaller than normal (25), suggesting that the mutants were also defective in virus production. This was confirmed by subsequent findings of decreased levels and rates of Sindbis production by endocytosis mutants (26). We have pursued these observations and in this paper we show that a single genetic lesion results in impairment both of ATP-dependent endosomal acidification and of Golgi apparatus-associated functions. A preliminary report of this work has been presented (29).

MATERIALS AND METHODS

Materials: Diphtheria toxin was from Dr. William H. Habig (Bureau of Biologics, Food and Drug Administration, Bethesda, MD) and from List Biological Laboratories Inc. (Campbell, CA); Pseudomonas exotoxin was provided by Dr. Stephen Leppla (U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD) and ricin was provided by Dr. Richard J. Youle (National Institute of Mental Health, Bethesda, MD); modeccin was purchased from Pierce Chemical Co. (Rockford, IL). Ouabain, ATP, thioguanine, fluorescein isothiocyanate-dextran (M_r >65,000), Percoll, galactose oxidase, and sodium lauryl sulfate (L-5750) were from Sigma Chemical Co. (St. Louis, MO); polyethylene glycol-1000 was from J. T. Baker Chemical Co. (Phillipsburg, NJ). Actinomycin D was from Aldrich Chemical Co. (Milwaukee, WI). [5,6-^{3}H]Uridine (40–60 Ci/mmol), [^{35}S]methionine (1,000–1,500 Ci/mmol), D-[2-^{3}H]mannose (10–20 Ci/mmol), D-[6-^{3}H]glucosamine (40 Ci/mmol), D-[1-^{3}H]galactose (10 Ci/mmol), L-[5,6-^{3}H]fucose (70 Ci/mmol), and NaB^3H_4 (20 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL); [9,10-^{3}H]palmitic acid (10–30 Ci/mmol) and ^{125}I (carrier-free, ~17 Ci/mg) were from New England Nuclear (Boston, MA). Monensin, nigericin, neuraminidase (*Vibrio cholerae*), lactoperoxidase, and bovine serum albumin were from Calbiochem-Behring Corp. (San Diego, CA). Pansorbin and Staph-A-Sorb (formalin-fixed *Staphylococcus aureus* cells, 10% [wt/vol]) were purchased from Calbiochem and Schwarz/Mann (Spring Valley, NY), respectively.

Cells: The isolation of the parent cells, WTB (32), and of the endocytosis-defective mutants DTF 1-5-1 and DTG 1-5-4 (25) has been previously described. The mutant Pro$^-$^{5}Lec2.6A (characterized in reference 33) was provided by Dr. Pamela Stanley (Albert Einstein College of Medicine, Bronx, NY). For studies of genetic complementation, spontaneous variants of WTB and DTG 1-5-4 resistant to ouabain and thioguanine were isolated using previously described strategies (32): cells were plated at a density of 1×10^6 cells/100-mm dish in growth medium containing 2 mM ouabain. The medium was replaced after 3–4 d, then once weekly. Individual colonies (1–4 from 10^6 cells) were picked and cloned as previously described (31); cloned variants were tested for plating efficiency in 2 mM ouabain. Ouabain-resistant cells were plated at 1×10^6 cells/100-mm dish in growth medium containing 5 μg/ml thioguanine; medium was replaced weekly. Colonies (2–18 from 10^6 cells) were picked and cloned, and the cloned variants were tested for both plating efficiency in ouabain and sensitivity to HAT medium (Dulbecco's modified Eagle medium containing 5% fetal bovine serum, 15 μg/ml hypoxanthine, 0.2 μg/ml aminopterin, and 5 μg/ml thymidine (34), supplemented with 11.5 μg/ml proline). Only those clones that yielded no survivors after plating of 1×10^6 cells in HAT medium were used in hybridizations.

Ricin-resistant variants were selected from the ouabain-resistant and thioguanine-resistant variant of DTG 1-5-4 (DTG 1-5-4-122). Cells were plated at 6×10^5/100-mm dish; then 8 h after plating the medium was replaced with growth medium containing 10% fetal bovine serum and 10 ng/ml ricin. After incubation with the toxin for 48 h, the medium was replaced with growth medium without ricin, and this medium was changed every 4 d. One colony from each dish (four total from 1.2×10^6 cells) was picked and cloned. Resistance to ouabain and thioguanine was maintained in the ricin-resistant variants.

Cell–Cell Fusion: The Oua^{Res}, Tg^{Res} variants were fused with the original Oua^{Sens}, Tg^{Sens} lines, and hybrids were selected in HAT medium containing 2 mM ouabain. Cells were plated 24 h before fusion at densities of $5–8 \times 10^5$ cells/35-mm well in six-well trays. Hybridization was carried out using a modification of the Ca^{++}-free procedure described by Schneiderman et al. (35): after rinsing the cells three times with growth medium lacking both serum and Ca^{++} and thoroughly removing the final rinse, 1 ml of a one-to-one mixture of polyethylene glycol (PEG 1000, J. T. Baker, Co.) and growth medium without Ca^{++} and serum was added gently to each well and the tray was swirled for 30 s. The polyethylene glycol mixture was then removed and the cells were gently washed three times with Ca^{++}- and serum-free growth medium, and then incubated for 30 min at 34°C in this same medium supplemented with 0.2% bovine serum albumin. At this time the medium was replaced with standard growth medium and the cells were returned to 34°C.

About 16 h after fusion, cells were harvested by trypsinization. For determination of viability, cells were plated in growth medium; for selection of hybrids, cells were plated in HAT medium containing 2 mM ouabain and supplemented with proline and 10% fetal bovine serum. Medium was replaced after 3–4 d and then once weekly until colonies containing several hundred cells were visible. These colonies were either picked individually and cloned, or the entire population of cells on a dish was harvested by trypsinization and cloned. ~10% of the original cells survived the polyethylene glycol procedure and 10% of these survivors were viable in HAT medium plus ouabain.

Secretion of Acid Hydrolases: Release of acid hydrolases into the medium was measured as previously described (25).

In Vitro Acidification Assay: Fluorescein (FITC)–dextran-loaded endosomes and lysosomes, isolated from wild type or mutant Chinese hamster ovary (CHO)[1] cells, were assayed for ATP-dependent acidification using a modification of previously published procedures (13). Cells were plated in 150-mm Falcon tissue culture dishes (Becton, Dickinson & Co., Oxnard CA) and grown at 34°C until confluent (4–9 d, depending on the cell line). To label endosomes, monolayers were incubated in growth medium containing 3.5–5.0 mg/ml dialyzed FITC-dextran for 15–30 min at 34°C. To label lysosomes, monolayers were incubated as above for 45 min at 34°C, washed three times with warm PBS, and then incubated an additional 1–2 h in medium without added FITC-dextran. The dishes were then placed on ice and washed extensively with cold HEPES-saline (120 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4). Monolayers were scraped from the dishes and lysed according to the method of Harms et al. (36) by disruption with a tight-fitting Dounce homogenizer. Percoll (in 0.25 M sucrose) was added to a postnuclear supernatant (750 *g*, 10 min) to a final concentration of 27% (ρ = 1.066 g/cm^3) and the mixture was

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; Man 6-P, mannose 6-phosphate; pfu, plaque-forming unit; VSV, vesicular stomatitis virus.

centrifuged at 4°C using either a Beckman Ti70 rotor (Beckman Instruments, Inc., Fullerton, CA) (20,000 *g*, 2 h) (13) or a Sorvall SV 288 rotor (DuPont Instruments, Sorvall Biomedical Div., Newtown, CT) (25). 1-ml fractions were collected, and the distribution of fluorescein and of lysosomal marker enzymes (β-hexosaminidase and β-galactosidase) was determined (37).

Changes in the internal pH of FITC–dextran-filled endosomes and lysosomes were estimated from changes in fluorescence intensity, which for FITC-dextran decreases as a function of decreasing pH (18, 38). Peak tubes corresponding to the endosome or lysosome fractions were pooled and 0.2 ml diluted into 2.8 ml of isotonic buffer (usually 125 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, and 10 mM HEPES, pH 7.5). ATP, ionophores, and inhibitors were added at the indicated concentrations. Fluorescence measurements were made at ambient temperature using a Perkin-Elmer Model 512 double-beam spectrofluorometer (Perkin-Elmer Corp., Norwalk, CT) at excitation and emission wavelengths of 485 and 515 nm, respectively (13). Relative changes in pH were determined from a standard curve of fluorescence intensity vs. pH (18). As an internal control, the fluorescence intensity in each sample was measured after the addition of 1 μM nigericin in isotonic KCl medium; under these conditions the internal pH was assumed to equilibrate with that of the surrounding medium (*i.e.*, pH 7.4) (13).

Virus and Virus Infection: Stocks of Sindbis and vesicular stomatitis viruses were prepared and titered as previously described (25). Unless otherwise specified, experiments were performed with cultures grown to a density of 1.5–2.0 × 10^6 cells/60-mm dish; these cells were infected with 4 × 10^7 plaque-forming units (pfu) of virus in one ml of growth medium; after 1 h at 34°C, the medium was replaced with 2 ml of growth medium containing 2 μg/ml actinomycin D.

Metabolic Labeling: For labeling cells with either [^{3}H]uridine or [^{3}H]palmitic acid, we used standard growth medium (30) containing 5% fetal bovine serum. For labeling with other metabolic precursors, growth medium, altered as indicated and containing 5% dialyzed fetal bovine serum (Gibco Laboratories), was employed at 1 ml/60-mm dish. [^{35}S]Methionine: cells were washed three times in medium without methionine, then labeled for 5 min with 80 μCi [^{35}S]methionine, 0.38 μg/ml or for 1 h with 10 μCi [^{35}S]methionine, 1.2 μg/ml. Tritiated sugars: cells were washed three times in glucose-free medium supplemented with 5 mM sodium pyruvate, then preincubated in this medium for 15 min at 34°C before labeling. Incubation of cells with tritiated sugars was for 1 h in medium containing 0.1 mM glucose plus pyruvate (39); cells were labeled with 100 μCi/ml [6-^{3}H]glucosamine or 50 μCi/ml [1-^{3}H]galactose; for labeling with [^{3}H]mannose (25 μCi/ml) or [^{3}H]fucose (50 μCi/ml), nonradioactive mannose or fucose were added at 18 μg/ml or 1.67 μg/ml, respectively. To chase radioactivity, we removed medium, rinsed the cells once in standard growth medium and then returned them to 34°C in standard growth medium. At the end of the chase period, cells were rinsed three times with ice-cold PBS without divalent cations.

Surface Labeling: Iodination was performed by the method of Morrison (40). Cells in 60-mm dishes were placed on ice and rinsed three times with 3 ml of ice-cold PBS (Gibco Laboratories). 1 ml of this buffer containing 300 μCi of ^{125}I was added to each dish, followed by addition of 14.7 IU of lactoperoxidase. To start the reaction, we added 2 μl of hydrogen peroxide (diluted to 0.00075%) to the mixture, and repeated this at 1, 2, 3, and 4 min. After 10 min, the radioactivity was removed and the cells were rinsed three times with 3 ml of ice-cold PBS containing bovine serum albumin, 10 mg/ml.

For labeling of cell surface galactose residues with galactose oxidase-NaB^3H_4 (41), cells were rinsed three times with PBS with divalent cations plus 2 mM phenylmethylsulfonyl fluoride, then incubated 15 min at 34°C either in this buffer or in buffer to which was added 0.1 IU of neuraminidase. Cells were then rinsed once in PBS and incubated 15 min at 25°C in PBS either with or without 15 U of galactose oxidase. Cells were then rinsed, adjusted to pH 8.0, and incubated for 15 min at 25°C in PBS containing 1.25 mCi of NaB^3H_4. The reaction was stopped by rinsing the cells three times in pH 8.0 buffer containing bovine serum albumin, 10 mg/ml.

Immunoprecipitation of Sindbis Proteins: After metabolic or surface labeling, infected cells on 60-mm dishes were lysed with 1 ml of buffer A (42): 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.02% sodium azide, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 1% bovine serum albumin. To each cell extract was added 40 μl of Pansorbin or Staph-A-Sorb (in PBS, without divalent cations). After incubation on ice for 30 min the extract was centrifuged for 5 min in an Eppendorf model 5414 microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY); 4 μl of rabbit anti-Sindbis antiserum was added to the supernate and the samples were incubated overnight on ice. 40 μl of Pansorbin or Staph-A-Sorb was added to each; samples were incubated 30 min at 0°C, and centrifuged for 1 min in a microcentrifuge. The pellets were washed once with 1 ml of buffer A, twice with 1 ml of buffer A without bovine serum albumin, and once with 1 ml of PBS without divalent cations. The immunoprecipitated material was dissociated by heating the pellets for 5 min at 95°C in electrophoresis sample buffer (43) containing 3% SDS, but without reducing agents; the suspensions were centrifuged for 5 min in an Eppendorf microcentrifuge and the supernatant fluids were stored at −20°C.

For immunoprecipitation of virions from the extracellular fluid, the medium was removed and centrifuged 10 min at 1,000 *g*; an aliquot of 0.1 ml of 0.5 M Tris, pH 6.8, was added per milliliter of supernate; samples were precleared with Pansorbin or Staph-A-Sorb, and immunoprecipitated as described above. By eliminating reducing agents from the electrophoresis buffer used on first heating the immunoprecipitated material to 95°C (and adding those agents just before electrophoresis of the samples, see below) the amount of full-sized Sindbis glycoprotein E2 recovered from the cell extracts was markedly increased.

Polyacrylamide Gel Electrophoresis and Fluorography: Samples were thawed, and 2-mercaptoethanol and dithiothreitol were added to 1% and 10 mM, respectively. Samples were heated (95°C for 5 min) centrifuged, and electrophoresed on SDS–10% polyacrylamide gels as described by Laemmli (43), with the single modification that 3-mercaptoproprionic acid was present at 0.025% in the upper buffer (44). With this addition the viral glycoproteins formed much sharper bands. Gels were subjected to fluorography (45), using preflashed XAR-2 film (Eastman Kodak Co., Rochester, NY) (46). For quantitation of radioactivity, labeled areas of the gel were excised, rehydrated in 0.1 ml of water for 30 min at 25°C, and heated in 1 ml of NCS (Amersham Corp.) for 20 min at 55°C. After incubation of the samples overnight at 25°C, 10 ml of toluene-based scintillant was added for determination of radioactivity by liquid scintillation spectrometry.

Analysis of Sialic Acid on Sindbis Glycoproteins: Cells (1.5–2.0 × 10^6/60-mm dish) were infected with 4 × 10^7 pfu of Sindbis as described above; 6 h after infection the cells were labeled for 1 h with [6-^{3}H]glucosamine, and the label was chased for 1.5 h. Sindbis proteins were immunoprecipitated from the cell extracts and the purity of the labeled material was monitored by SDS PAGE and fluorography, as described above. Parallel immunoprecipitated pellets (containing antigen, antibody, and Staph-A-Sorb) were extracted with mixtures of chloroform, methanol, and water (47) to remove sugar, sugar phosphate, sugar nucleotide, and glycolipid. The protein residue was resuspended in 1 ml of 0.1 M HCl and heated for 50 min at 80°C to release neuraminic acid. After centrifugation at 1,000 *g* for 5 min the residue was heated in 1 ml of 2 M HCl for 2 h at 100°C. Products of both the mild and the strong acid hydrolyses were separated by descending paper chromatography (Whatman 3MM, Whatman Chemical Separation, Inc., Clifton, NJ) for 24 h in (3:2:1) *n*-butyl acetate/acetic acid/water (48). A portion of each lane was used to visualize internal standards (1 μmol each of *N*-acetylneuraminic acid, *N*-acetylglucosamine, glucosamine, and glucose) with silver nitrate (49). The remainder of each lane was cut into 1-cm slices; after addition of 0.5 ml of water and 4 ml of Liquiscint (National Diagnostics, Somerville, NJ), radioactivity was determined by liquid scintillation spectrometry.

Electron Microscopy: Cells were grown in 150-cm^2 flasks to densities of 1.5–2.0 × 10^7 cells/flask, then infected with Sindbis at 4 × 10^8 pfu/10 ml. Actinomycin D was omitted from these experiments to avoid its effects on cell morphology. 7 h after infection cells were harvested by trypsinization, washed once with medium plus serum and twice with medium without serum, and then resuspended in fixative containing 2% glutaraldehyde (Ladd Research Industries, Burlington, VT) and 2% formaldehyde (Ladd) in 0.1 M cacodylate buffer at pH 7.4. Cells were fixed for 3–4 h at room temperature, rinsed in cacodylate buffer containing 7% sucrose (sucrose buffer), and stored overnight at 4°C in sucrose buffer. Samples were postfixed in 2% osmium tetroxide in sucrose buffer for 1 h at room temperature, rinsed in sucrose buffer and stained en bloc for 2 h at room temperature with either 0.5% uranyl acetate in distilled water or 1% tannic acid in sucrose buffer. The suspensions were dehydrated through a graded series of ethanol and propylene oxide or acetone and embedded in Spurr's resin (50). Thin sections were cut with a diamond knife, mounted on bare copper grids, stained with Reynolds' lead citrate (51) and uranyl acetate, and examined in an JEOL 100C electron microscope (JEOL USA, Electron Optics Div., Peabody, MA).

RESULTS

Genetic Analyses of Mutant Cell Lines

DTG 1-5-4, like the mutants previously described (25), was isolated by first treating CHO cells with diphtheria toxin, and then screening the survivors for those cells defective in uptake of ligand via the mannose 6-phosphate (Man 6-P) receptor. In comparison with parental cells (WTB), DTG 1-5-4 exhibited increased resistance to Pseudomonas toxin and modeccin, as well as to diphtheria toxin, and increased sensitivity to ricin

(Table I). Man 6-P receptor-dependent uptake was <5% of normal in this mutant (Table I), and 90–95% of the endogenously synthesized acid hydrolyases (β-hexosaminidase, α-L-iduronidase, α-L-fucosidase, α-mannosidase, β-galactosidase) were secreted (data not shown). An identical phenotype was observed in DTG 1-5-4-122, a ouabain-resistant, thioguanine-resistant derivative of DTG 1-5-4 isolated for genetic studies.

In an attempt to isolate revertants, we selected variants of DTG 1-5-4-122 able to survive treatment with 10 ng/ml ricin for 48 h, (conditions that reduced the plating efficiencies of WTB and DTG 1-5-4-122 to 93% and 0.0003%, respectively). We obtained cells with normal sensitivity to diphtheria toxin, Pseudomonas toxin, and modeccin and with normal uptake via the Man 6-P receptor (Table I). Thus, the various alterations in endocytosis in DTG 1-5-4 appear to result from a single genetic defect.

DTG 1-5-4 was more severely affected than mutant DTF 1-5-1, another endocytosis mutant described in previous publications (25, 28), in which resistance to Pseudomonas toxin and modeccin was normal, and resistance to diphtheria toxin, sensitivity to ricin, secretion of acid hydrolases, and loss of Man 6-P receptor-dependent uptake were less pronounced. Results of complementation analyses of DTF 1-5-1 × DTG 1-5-4-122 hybrids indicated that the mutations in DTF 1-5-1 and DTG 1-5-4 were in different genes (Table II). For these analyses, hybrids generated by polyethylene glycol-mediated fusion were selected for markers unrelated to the endocytic defect (see Materials and Methods), cloned, and then tested for resistance to diphtheria toxin and Man 6-P receptor-dependent uptake. We found that toxin sensitivity and ligand uptake in DTF 1-5-1 × DTG 1-5-4-122 hybrids were restored to 90% of the levels measured in mutant × WTB hybrids.

Acidification of Endosomes and Lysosomes In Vitro

The phenotypes of DTF 1-5-1 and DTG 1-5-4 resemble that of cells treated with agents that inhibit acidification of endosomes and lysosomes. We examined organelle acidification in the mutants using endosomes and lysosomes isolated from cells that had been labeled with the pH-sensitive fluorochrome FITC-dextran (13, 27). As shown in Fig. 1, when WTB, DTF 1-5-1, or DTG 1-5-4 cells were labeled with a 15–30-min pulse of FITC-dextran, the labeled vesicles sedimented as a single peak of low density (ρ = 1.03 g/cm^3) on Percoll gradients. This low-density peak was defined as the endosomal fraction. After a 60-min chase, most of the internalized FITC-dextran in WTB and DTF 1-5-1 (Fig. 1, *A* and *B*) was found in a peak of much higher density (ρ = 1.10 g/cm^3); in WTB this is the position of the major peak of lysosomal marker enzymes (Fig. 1 *D*). In contrast, even after chase, 50% of the FITC-dextran remained in vesicles of low density in DTG 1-5-4; Fig. 1 *C* shows the distribution of label after a 1-h chase; no further movement of label from light to heavy peaks was observed when the chase periods were extended to 2 h. This low density "lysosome" peak of DTG 1-5-4 was slightly more dense than the endosome peak.

As observed previously with J774 macrophages and baby hamster kidney-21 fibroblasts (13), FITC–dextran-labeled endosomes and lysosomes from WTB could be shown to lower their internal pH in vitro. Upon the addition of 2 mM ATP, a rapid quenching of fluorescein fluorescence occurred; quenching was immediately reversed by agents that collapse transmembrane pH gradients, e.g., the Na^+, K^+-H^+ ionophore nigericin (1 μM) (Fig. 2). Acidification was completely inhibited by *N*-ethylmaleimide (1.0 mM) and the sulfhydryl-reactive reagent 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole, 25–50 μM (Fig. 3). Sensitivity of ATP-driven proton transport to these inhibitors has been previously observed with Golgi membranes (20), J774 endosomes, and bovine adrenal chromaffin granules (Galloway, C., G. Dean, I. Mellman, and G. Rudnick, unpublished results). Inhibitors of either the Na^+K^+-ATPase (vanadate) or the mitochondrial F_1F_0-ATPase (efrapeptin or NaN_3) had no effect on the acidification of WTB endosomes and lysosomes (data not shown).

Endosomes from mutant DTG 1-5-4 displayed little or no ATP-dependent acidification in vitro (Fig. 2). This lack of activity was not due to insufficient FITC-dextran in the endosomes, because the amount of fluorochrome present in the endosome peak from the mutant was well within the range of sensitivity of the assay, as determined using progressively

TABLE I

Endocytosis in WTB, Mutants, and Revertants

	WTB	DTG 1-5-4	DTG 1-5-4-122	Rev 123	Rev 211
Inhibition: EC_{50}, *ng/ml**					
Diphtheria toxin	40	1,000	700	50	30
Modeccin‡	2	>3,000	>3,000	1	1
Pseudomonas toxin	350	3,000	3,500	240	560
Ricin	140	3	2	60	90
Uptake, *cpm/μg of protein*§					
[^{35}S]Secretions	110	0	0	80	100

Rev, revertant.

* Inhibition of protein synthesis by the various toxins was assayed as previously described (25). EC_{50} is the dose required to inhibit protein synthesis to 50% of that measured in parallel samples of untreated cells.

‡ At 3,000 ng/ml modeccin, the highest dose tested, protein synthesis in DTG 1-5-4 and DTG 1-5-4-122 remained 100% of that measured in untreated cells.

§ Uptake via the Man 6-P receptor was measured using ammonia-induced secretions from WTB cells grown in the presence of [^{35}S]methionine (25); 7×10^5 cpm of these secretions were added in doses of 2 ml to cells grown on 60-mm dishes. Uptake was assayed as previously described (25); total cell protein was measured by the Lowry method (52). Values presented are corrected for uptake measured in the presence of 5 mM Man 6-P; values for nonspecific uptake were similar in all cell types tested (12 cpm/μg protein).

TABLE II

Endocytosis in WTB, Mutants, and Cell–Cell Hybrids

Cells	Diphtheria toxin inhibition*	Man 6-P uptake
	EC_{50}, *ng/ml*	*cpm/μg protein*
WTB	25	147
DTF 1-5-1	300	19
DTG 1-5-4-122	700	3
DTF 1-5-1 × DTG 1-5-4-122	40	91

Inhibition of protein synthesis by toxin and uptake of ^{35}S secretions by the Man 6-P receptor were measured as described in Table I. Cloned hybrids from two independent fusions were examined.

* We have observed significant variation in the sensitivities of the mutants, without corresponding changes in the sensitivity of WTB, to different preparations of diphtheria toxin. EC_{50}'s have ranged from 30, 2,100, and 5,500 ng/ml for WTB, DTF 1-5-1, and DTG 1-5-4, respectively, with the original preparation used in isolation of the mutants, to 15, 120, and 300 ng/ml for the same three cell types. Increased potency of toxin for the mutants appeared to be proportional to the level of nicked toxin present in the preparation, as determined by electrophoresis under reducing vs. nonreducing conditions on SDS polyacrylamide (11%) gels. No other parameters measured in the mutants were found to vary.

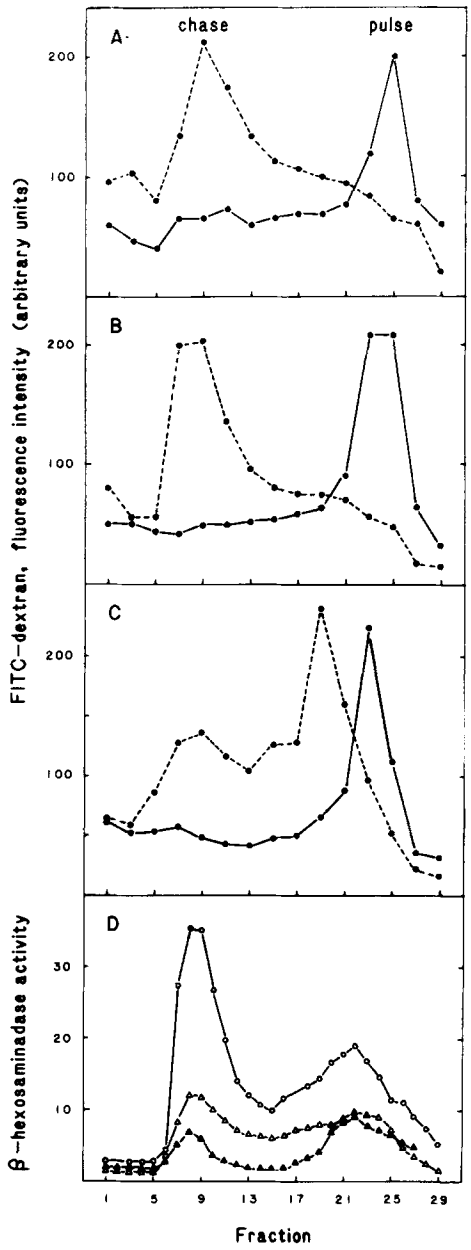

FIGURE 1 Separation of endosomes and lysosomes from WTB and mutant CHO cells by Percoll density-gradient centrifugation. To label endosomes, cells were incubated at 34°C in medium containing 3.0–6.0 mg/ml dialyzed FITC-dextran for 15–30 min ("pulse"). To label secondary lysosomes, FITC-dextran-pulsed cells were washed with PBS and incubated at 34°C for an additional 60 min in marker-free medium ("chase"). Cells were then homogenized and centrifuged in self-forming Percoll gradients as described in Materials and Methods. 1-ml fractions were collected and FITC-dextran fluorescence determined in every other tube. Sedimentation profiles of endosomes (●——●) and lysosomes (●-----●) are shown for WTB (*A*), DTF 1-5-1 (*B*), and DTG 1-5-4 (*C*). Distribution of a lysosomal marker enzyme, β-hexosaminidase, is shown in *D*: WTB (○), DTF 1-5-1 (△), DTG 1-5-4 (▲). Enzyme activity, expressed in arbitrary units, was determined in gradient fractions derived from homogenates containing similar amounts of cell protein.

smaller aliquots of labeled WTB endosomes.

Acidification of endosomes from DTF 1-5-1 was only partially affected (Fig. 2). The ATP-dependent decrease in pH was reduced in comparison with that observed with WTB endosomes; however, the overall pH gradient (determined by the increase in fluorescence after addition of nigericin) was similar to that of WTB endosomes. In contrast, both total and ATP-induced pH gradients in DTG 1-5-4 endosomes were reduced relative to controls. These results were explained by measurements of endosomal pH before addition of ATP. As shown in Table III, endosomes of DTF 1-5-1 were slightly acidic as isolated; thus, the total pH gradient measured in endosomes from this mutant after addition of nigericin reflected mainly the initial pH of those organelles. The inhibition of ATP-dependent acidification of endosomes from DTF 1-5-1 was not due to a decreased affinity of the proton pump for ATP, in that experiments performed over a range of ATP concentrations (0.01–5.0 mM) indicated that proton pumping in both WTB and mutant endosomes was half-maximal at 0.2 mM.

FITC-dextran-labeled lysosomes from both DTF 1-5-1 and DTG 1-5-4 cells acidified in vitro nearly as well as WTB lysosomes (Fig. 2). Both "heavy" and "light" lysosomal peaks from DTG 1-5-4 cells (Fig. 1) were capable of ATP-driven acidification.

Endosomes isolated from DTF 1-5-1 × DTG 1-5-4-122 hybrids (Table III) and from revertants of DTG 1-5-4-122 (Fig. 3) exhibited ATP-driven acidification similar to endo-

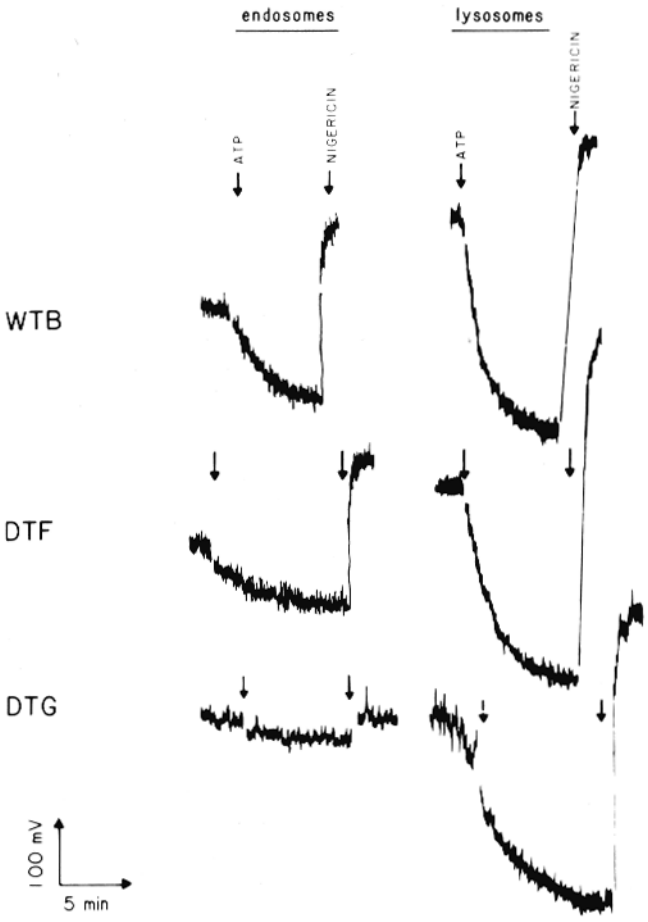

FIGURE 2 Acidification of endosomes and lysosomes from WTB and mutant CHO cells. Endosomes and lysosomes from WTB, DTF 1-5-1, and DTG 1-5-4 cells were labeled with FITC-dextran and separated on Percoll density gradients. Peak fractions were pooled and diluted into isotonic KCl buffer (see Materials and Methods), and acidification was measured as a decrease in FITC fluorescence after the addition of 5 mM ATP. Nigericin (in ethanol) was added to a final concentration of 1 μM to collapse transmembrane pH gradients. Addition of ethanol (<1%) alone had no effect.

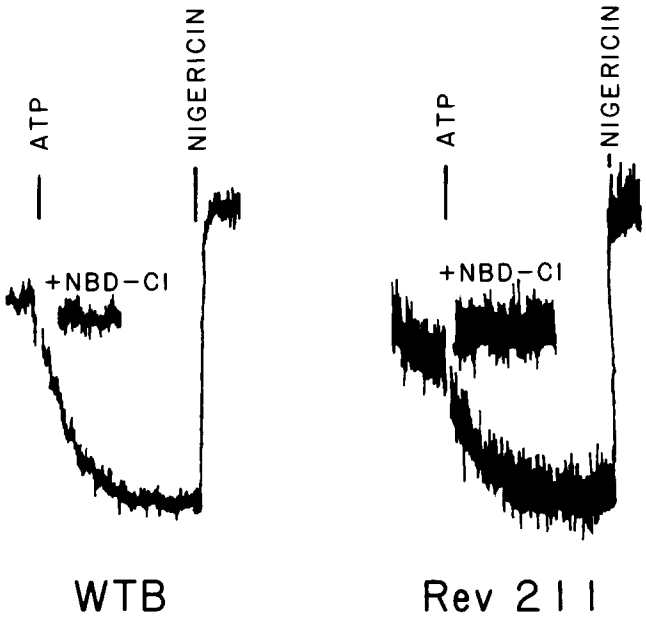

FIGURE 3 Acidification of endosomes from WTB and a revertant of DTG 1-5-4-122. Endosomes from WTB and revertant (Rev) 211 cells were labeled with FITC-dextran and isolated by Percoll gradient centrifugation. Acidification was assayed as described in the legend to Fig. 2. Addition of 25 μM 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole before the addition of ATP inhibited ATP-dependent acidification in both WTB and Rev 211 endosomes.

TABLE III

In Vitro Acidification of Endosomes from WTB, Mutant, and Hybrid Cells

	WTB	DTF 1-5-1	DTG 1-5-4	DTF 1-5-1 × DTG 1-5-4-122
Initial pH	7.0	6.8	7.2	7.2
Δ pH, total	0.71	0.73 ± 0.02	0.33 ± 0.10	0.56
Δ pH, ATP-dependent	0.36	0.17 ± 0.04	0.13 ± 0.07	0.36

Each value was determined relative to an internal control, i.e., the fluorescence intensity of each sample at pH 7.4 (see Materials and Methods). Initial pH was measured with the endosomal fractions as isolated, without addition of ATP; total Δ pH was measured after addition of ATP; ATP-dependent Δ pH was calculated as [Δ pH, total − (pH 7.4 − initial pH)].

somes from WTB. Thus, restoration of endocytosis (Tables I and II) correlated with restoration of acidification activity in isolated endosomes.

Virus Infection of Mutants

DTG 1-5-4, like DTF 1-5-1 (25) and RPE.28 and RPE. 44 (26), two other CHO cell mutants defective in endosome acidification (27), exhibited increased resistance to Sindbis virus and vesicular stomatitis virus (VSV). Monolayers of DTF 1-5-1 and DTG 1-5-4 showed one-sixth the number of viral plaques found on monolayers of WTB cells. Using synthesis of Sindbis RNA as a measure of successful viral penetration, we found that infection of both DTF 1-5-1 and DTG 1-5-4 was reduced from two- to fivefold in comparison with WTB, at viral multiplicities ranging from 1 to 64 pfu/cell (Fig. 4). Infection of WTB cells in the presence of NH_4Cl (10 mM) reduced Sindbis RNA synthesis to <5% of the levels measured in untreated cells.

Decreased efficiency of viral penetration is consistent with the decreased efficiency of Sindbis plaque formation, in that initiation of a plaque presumably requires successful infection of a cell by a single virion. But, in addition, the viral plaques formed on DTF 1-5-1 and DTG 1-5-4 were much smaller than normal, especially in the case of DTG 1-5-4. For this reason we examined synthesis and release of Sindbis virus by the mutants. Using viral multiplicities at which the mutants were successfully infected (≥20 pfu/cell), we found that synthesis of viral proteins in DTG 1-5-4 and DTF 1-5-1 equaled that in the parent, but release of virions was only 5% and 20% of the parental level, respectively (Fig. 5). Normal release of virus was obtained from the DTF 1-5-1 × DTG 1-5-4 hybrids (Fig. 5). Revertant 123 from DTG 1-5-4-122 showed full restoration of Sindbis release, while revertant 211 released virus at 35% of the parental level (Fig. 6). The results shown are from experiments in which viral proteins and virions were immunoprecipitated from cell extracts and the extracellular fluid; similar results were obtained on electrophoresis of whole extracts of infected cells and of virions separated from the medium by centrifugation.

The decreased production of Sindbis virus from the mutants does not reflect a simple delay in virion release. When infected cells were incubated with [^{35}S]methionine and the radiolabel was chased for various intervals, we found that the onset of viral release from both mutants and the parent occurred at 35–40 min after synthesis. The amount of labeled virus released from the three cell types then increased linearly with time for 3–4 h. In contrast, VSV release from the mutants (infected at >20 pfu/cell) was delayed, but after 3–4 h of chase [^{35}S]methionine-labeled VSV was found at normal levels outside the mutant cells (data not shown).

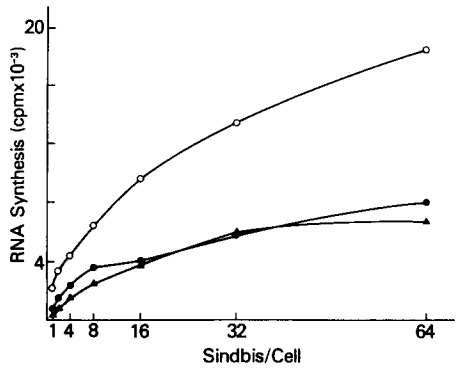

FIGURE 4 Effect of viral multiplicity on Sindbis infection of WTB, DTF 1-5-1, and DTG 1-5-4. Cells (grown in 24-well trays to 1.5 × 10^5 cells/well) were incubated with the indicated amounts of virus (defined as pfu) for 1.5 h at 34°C, then the medium was replaced with growth medium containing 2 μg/ml actinomycin D. After an additional incubation for 30 min at 34°C, this was replaced with growth medium containing actinomycin D, 15 mM NH_4Cl (to prevent further infection), and [^{3}H]uridine (12 μCi/ml). After 2.5 h the trays were put on ice, the wells were rinsed twice with 2 ml of PBS plus 10 mM uridine, a solution of 0.1% trypsin, 1 mM EDTA was added, the trays were placed at 34°C for 5 min, then aliquots of released cells were spotted on strips of Whatman 3 paper; these strips were soaked in 10% trichloroacetic acid (0°C), rinsed in ethanol, dried, and counted all as described for assays of protein synthesis (8). All assays were run in triplicate and values were corrected for incorporation in uninfected cells (750 cpm). (○) WTB, (●) DTF 1-5-1, (▲) DTG 1-5-4.

Maturation of Viral Glycoproteins

We pursued our examination of Sindbis virus release using DTG 1-5-4, the more affected of the two mutants.

In Figs. 5 and 6 it may be noted that Sindbis glycoproteins E1 and E2 isolated from DTG 1-5-4 migrated slightly more rapidly than the corresponding proteins from WTB. As shown in Fig. 7, these differences were not observed until ~20 min after synthesis of the glycoproteins. Cells infected with Sindbis were incubated for 5 min with [^{35}S]methionine, then the label was chased for various times; at 20 min, E1 from WTB cells moved more slowly than at earlier times, whereas no change in the mobility of E1 from DTG 1-5-4 was observed through 120 min. No differences were seen in the mobilities of the precursor pE2 isolated from the two cell types; however,

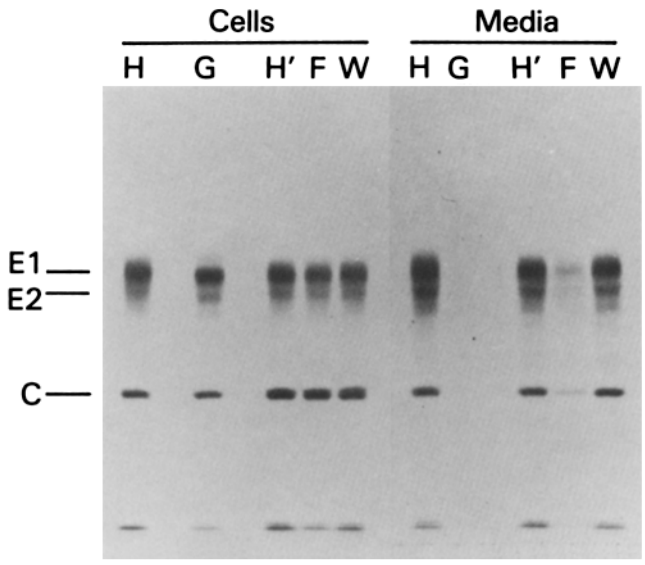

FIGURE 5 Synthesis and release of Sindbis virus in mutants, hybrids, and WTB. Cells (grown to 1.5–2 × 10^6 cells/dish on 60-mm dishes) were infected with 4 × 10^7 pfu of Sindbis virus; after 5 h, cells were labeled for 1 h with [^{35}S]methionine, 10 μCi/ml, then radioactivity was chased for 2 h. Viral proteins were immunoprecipitated from media and cell extracts; one-half of each sample was used in electrophoresis. Details of the procedures used are given in Materials and Methods. The figure is a composite of two fluorographic exposures of a single gel; the area under *Cells* was exposed for 24 h, that under *Media* for 4 d. (Lane *H*) DTF 1-5-1 × DTG 1-5-4-122 #2-2; (lane *G*) DTG 1-5-4; (lane *H'*) DTF 1-5-1 × DTG 1-5-4-122 #3; (lane *F*) DTF 1-5-1; (lane *W*) WTB. *E1*, *E2*, and *C* indicate the positions of the two viral glycoproteins and the nucleocapsid protein, respectively.

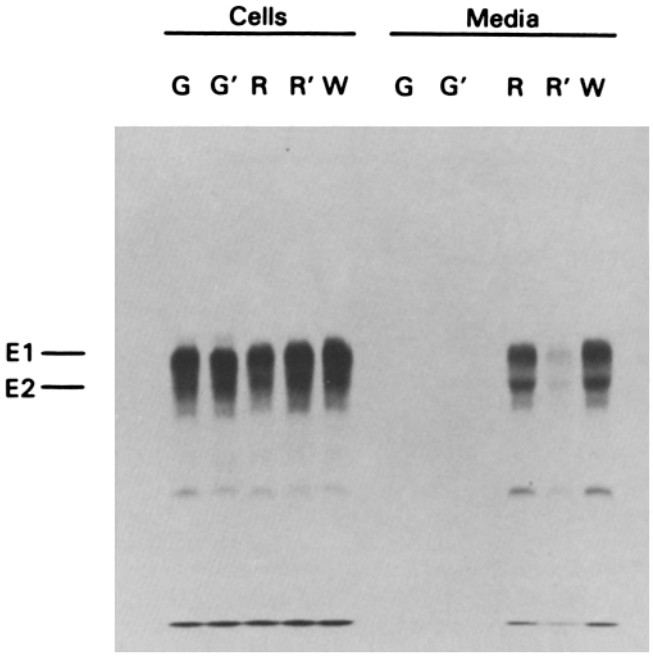

FIGURE 6 Synthesis and release of Sindbis virus in WTB, DTG 1-5-4, and revertants of DTG 1-5-4-122. Conditions of infection and labeling are described in the legend of Fig. 5. One-sixth of the immunoprecipitate from each cell extract and one-half of each immunoprecipitate from the medium were electrophoresed on the gel. The fluorograph was exposed for 4 d. The low level of capsid protein obtained in this experiment as contrasted to that seen in Fig. 5 appears to reflect different degrees of adsorption of capsid protein to different preparations of *S. aureus*, used in immunoprecipitation. (Lane *G*) DTG 1-5-4; (lane *G'*) DTG 1-5-4-122; (lane *R*) Revertant 123; (lane *R'*) Revertant 211; (lane *W*) WTB.

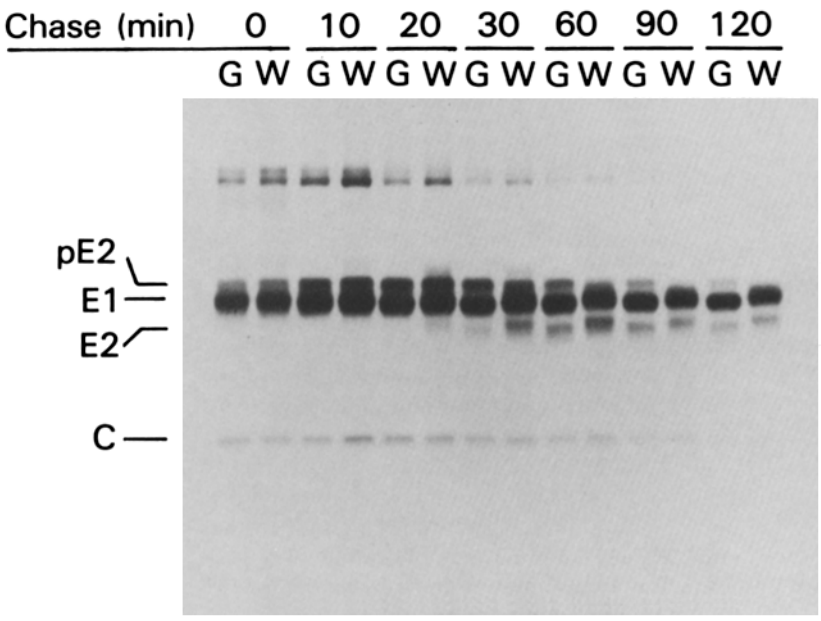

FIGURE 7 Maturation of Sindbis glycoproteins in DTG 1-5-4 and WTB. At 5 h after infection with Sindbis virus, cells (grown and infected as described in Fig. 5) were incubated with [^{35}S]-methionine (80 μCi/ml) for 5 min, then radioactivity was chased for the times indicated in the figure. One-fourth of the immunoprecipitate obtained from each sample was electrophoresed on the gel, and the fluorograph was exposed for 24 h. The electrophoretic mobility of the transient high molecular weight proteins suggests that they may be related to B protein, a nonstructural, nonglycosylated Sindbis protein containing both pE2 and E1 (53). (Lane *G*) DTG 1-5-4; (lane *W*) WTB.

cleavage of pE2 to E2 was delayed (30 vs. 20 min) in the mutant and some pE2 persisted in the mutant through 120 min of chase, whereas essentially all of the pE2 had disappeared from the parent at 60 min. At all time points E2 from the mutant migrated more rapidly than E2 from the parent.

These results suggested that the Sindbis glycoproteins from DTG 1-5-4 lacked some posttranslational modification(s). On labeling infected cells with the appropriate metabolites, we found that mannosylation, fucosylation, and acylation of the Sindbis glycoproteins occurred to similar extents in mutant and parent cells (Fig. 8). In contrast, galactosylation of these proteins was reduced in DTG 1-5-4 (Fig. 9); incorporation of [1-^{3}H]galactose in E1 and E2 in the mutant was <15% of that measured in WTB cells, whereas incorporation of [^{35}S]methionine in parallel cultures of the mutant was greater than parental levels. These results were confirmed by experiments in which intact Sindbis-infected DTG 1-5-4 and WTB were reduced with NaB^3H_4 after treatment with galactose oxidase. Both E1 and E2 immunoprecipitated from the parental cells were labeled, and incorporation of radioactivity into these proteins was (*a*) increased by incubation with neuraminidase prior to galactose oxidase and (*b*) totally dependent on treatment with galactose oxidase. No radioactivity was detected in Sindbis glycoproteins from DTG 1-5-4 even after treatment with neuraminidase (data not shown).

Decreased galactosylation is not a general phenomenon in DTG 1-5-4; also shown in Fig. 9 are extracts of mutant and parent cells that had been infected with vesicular stomatitis virus then incubated with [1-^{3}H]galactose or [^{35}S]methionine. Incorporation of galactose into VSV glycoprotein G in DTG

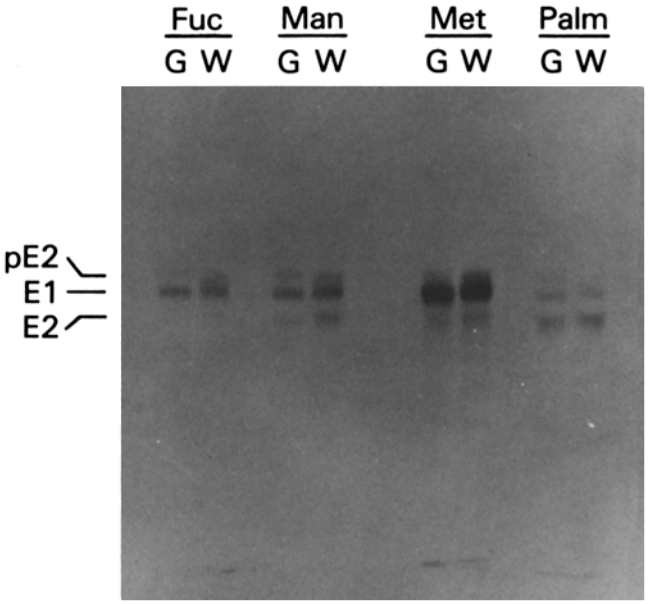

FIGURE 8 Incorporation of fucose, mannose, methionine, and palmitate into Sindbis proteins in DTG 1-5-4 and WTB. Cells grown and infected with Sindbis as described in Fig. 5 were incubated with the following radioactive precursors at 5 h after infection: (*Fuc*) [5,6-^{3}H]fucose (50 μCi/ml); (*Man*) [2-^{3}H]mannose (25 μCi/ml); (*Met*) [^{35}S]methionine (10 μCi/ml); (*Palm*) [9,10,-^{3}H(N)]palmitic acid (25 μCi/ml); details of labeling procedures are given in Materials and Methods. After 1 h, the radioactivity was chased for an additional 1.5 h. One-fourth of the immunoprecipitates from tritium-labeled samples and one one-hundredth of the immunoprecipitates containing ^{35}S were electrophoresed on the gel. The fluorograph was exposed for 8 d. (Lane *G*) DTG 1-5-4; (lane *W*) WTB.

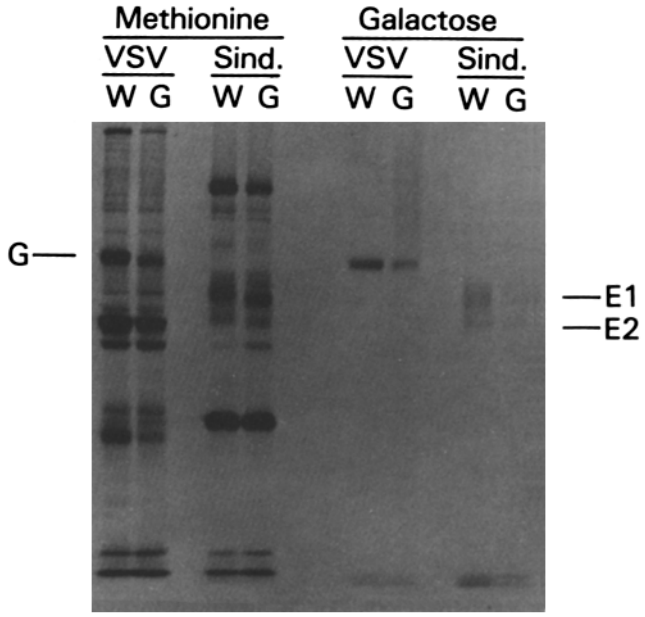

FIGURE 9 Galactosylation of Sindbis and VSV glycoproteins in DTG 1-5-4 and WTB. Cells grown on 60-mm dishes to ~2 × 10^6 cells/dish were infected with 4 × 10^7 pfu of each virus in 1 ml; 5.5 h after infection cells were incubated with either [^{35}S]methionine (10 μCi/ml) or [1-^{3}H]galactose (50 μCi/ml). Cells were labeled for 60 min, then the label was chased for 70 min. At this time cells were rinsed, harvested by scraping, and centrifuged, and the pellets were dissolved directly in gel buffer. One-fourth of the extracts from cells labeled with [^{3}H]galactose and one-sixtieth of those labeled with [^{35}S]methionine were electrophoresed on the gel. The fluorograph was exposed for 8 days. *W*, WTB; *G*, DTG 1-5-4.

1-5-4 ranged from 105% to 140% of that measured in WTB cells after correction for the relative incorporation of methionine into G protein in the two cell types. Decreased galactosylation of Sindbis glycoproteins is genetically related to the endocytic defect in DTG 1-5-4; as shown in Fig. 10 incorporation of [1-^{3}H]galactose into Sindbis was partially restored in revertant 211 and fully restored in revertant 123.

As would be predicted from the reduced galactosylation of Sindbis glycoproteins in DTG 1-5-4, sialylation of those proteins was also reduced in the mutant. After labeling of infected cells with [6-^{3}H]glucosamine, viral proteins were immunoprecipitated from DTG 1-5-4, WTB, and Pro$^-$^{5}Lec2.6A, a mutant that does not sialylate asparagine-linked oligosaccharides (33). The proteins were subjected to mild and then strong acid hydrolysis to remove sialic acid, and then all remaining sugars. Sugars were separated by chromatography: no differences were observed in the amount of [^{3}H]glucosamine from the three cell types; the radioactivity measured as sialic acid was 345, 20, and 0 cpm per 10,000 cpm of glucosamine recovered from WTB, DTG 1-5-4, and Pro$^-$^{5}Lec2.6A, respectively. The reduced sialylation of E1 and E2 in DTG 1-5-4 probably accounts for the different electrophoretic mobilities of those glycoproteins isolated from mutant and parent cells.

The major cellular glycoproteins in DTG 1-5-4 appeared to be unaffected in terminal glycosylation. Uninfected mutant and parent cells were incubated for 1 h with [2-^{3}H]mannose or [1-^{3}H]galactose, radioactivity was chased for 1.5 h, and total extracts of the cells were prepared and electrophoresed on SDS–polyacrylamide gels. No differences were observed between extracts of mutant and parent with respect to either the electrophoretic mobilities of the [^{3}H]mannose-containing

proteins or the amount of [^{3}H]galactose incorporated into the various radiolabeled bands (data not shown).

The amounts of surface-associated viral glycoproteins were compared in mutant and parent cells using lactoperoxidase-catalyzed iodination at 0°C. As shown in Fig. 11*A*, glycoproteins E1 and E2 were found on DTG 1-5-4 at 30% of the amounts measured for WTB. A small amount of the precursor glycoprotein pE2 also was iodinated on the mutant. No iodinated pE2 was observed on WTB.

The alterations in maturation of Sindbis in DTG 1-5-4 resemble, at least qualitatively, results reported for cells treated with monensin subsequent to viral infection (54, 55). We have repeated these studies using WTB treated with 1 and 10 μM monensin; our results are summarized in Table IV. Note that treatment of parental cells with monensin also resulted in the appearance of pE2 at the cell surface (Fig. 11*B*).

Sindbis-infected WTB and DTG 1-5-4 cells were examined by electron microscopy before and after treatment with 10 μM monensin. In untreated WTB cells (Fig. 12*A*) the Golgi apparatus consisted of stacks of five to seven saccules. Short cisternae studded with viral nucleocapsids were occasionally seen near the Golgi saccules. Numerous single virions could

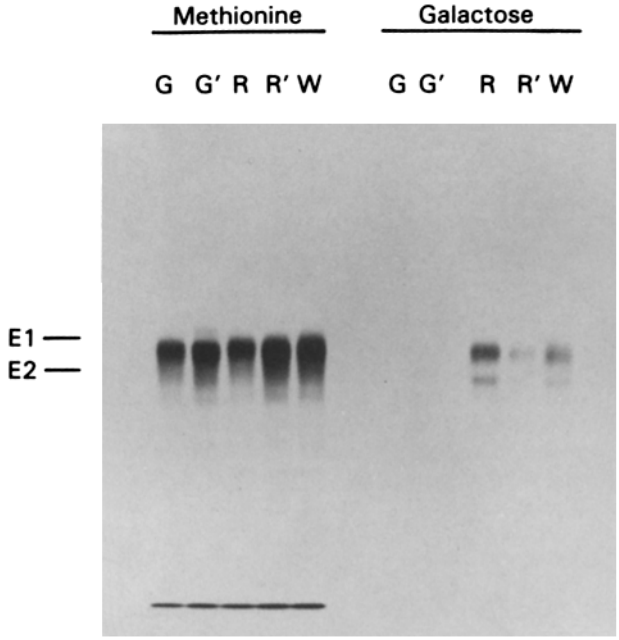

FIGURE 10 Galactosylation of Sindbis glycoproteins in revertants of DTG 1-5-4-122. After 5 h of infection, cells grown and infected as described in Fig. 5 were incubated for 1 h with either [^{35}S]-methionine (10 μCi/ml) or [1-^{3}H]galactose (50 μCi/ml); the label was then chased for 1 h. Viral proteins were immunoprecipitated, one-fortieth of the immunoprecipitates from methionine-labeled cells and one-half from galactose-labeled cells were electrophoresed on the gel. The fluorograph was exposed for 4 d. (lane *G*) DTG 1-5-4; (lane *G'*) DTG 1-5-4-122; (lane *R*) revertant 123; (lane *R'*) revertant 211; (lane *W*) WTB.

TABLE IV

Comparison of Viral Maturation in Mutant and Monensin-treated Cells

	Fraction of WTB response		
		WTB-Monensin, μM	
	DTG 1-5-4	1	10
Sindbis			
pE2 → E2*	0.70	0.38	0.17
Fucosylation‡	0.88	0.67	0.60
Galactosylation§	0.08	0.36	0.10
Transport to Cell Surface‖	0.30	0.09	0.03
Release¶	0.05	0.05	0.03
VSV			
Galactosylation§	1.4	1.14	0.07

All values were obtained by excising and counting relevant bands from SDS polyacrylamide gels (see Materials and Methods). Values reported for galactosylation were obtained by electrophoresis of total infected cell extracts; other values were obtained by electrophoresis of viral proteins after immunoprecipitation. Comparison of values for Sindbis galactosylation using immunoprecipitates vs. whole infected cell extracts revealed no significant differences.

* 15-min pulse with [^{35}S]methionine; 90-min chase. Ratio of E2/E1.

‡ 1-h pulse with [^{3}H]fucose or [^{35}S]methionine, 1.5-h chase. Ratio of [^{3}H]/[^{35}S]glycoproteins.

§ 1-h pulse with [^{3}H]galactose or [^{35}S]methionine, 1.5-h chase. Ratio of [^{3}H]/[^{35}S]glycoproteins.

‖ Lactoperoxidase-catalyzed iodination at 0°C, 7 h after infection. Total glycoproteins.

¶ 1-h pulse with [^{35}S]methionine, 2-h chase. Ratio of extracellular/intracellular E1.

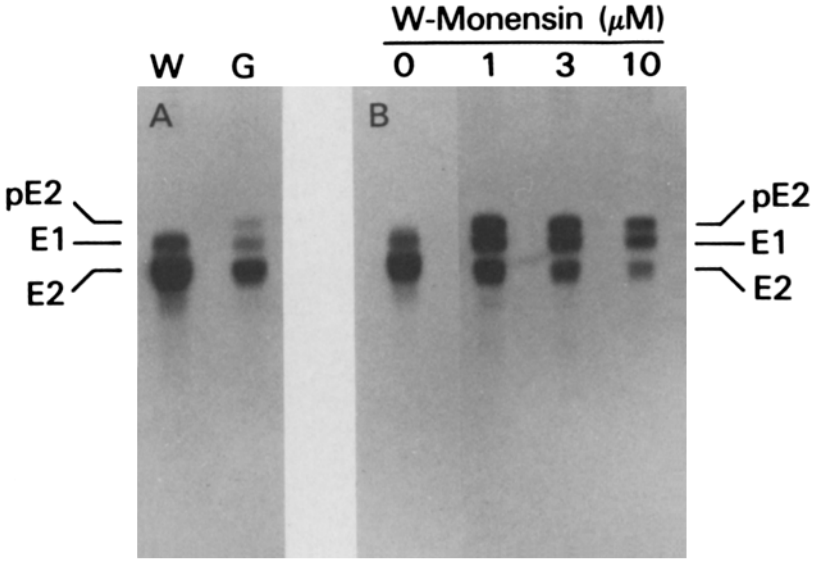

FIGURE 11 Surface-associated Sindbis proteins in WTB, DTG 1-5-4, and WTB cells treated with monensin. Cells grown and infected with Sindbis virus as described in Fig. 5 were placed on ice 7 h after infection. Lactoperoxidase-catalyzed iodination was performed at 0°C as described in Materials and Methods. One-fourth of the protein immunoprecipitated from each cell extract was electrophoresed on the gel. (A) Iodinated proteins from WTB (lane *W*) and DTG 1-5-4 (lane *G*). The fluorograph was exposed for 1 d. (B) Iodinated viral proteins from WTB treated with the indicated concentrations of monensin 2 h after infection with Sindbis virus. This is a composite of two fluorographic exposures of a single gel; the area under *0* was exposed for 8 h, the remainder was exposed for 8 d.

be seen budding at the cell surface (Fig. 12 *B*). In the monensin-treated cells, as has been previously reported (54, 56), the Golgi saccules were swollen (Fig. 12 *C*), and some of the saccules had nucleocapsids bound to their surfaces (Fig. 12, *C* and *D*). Although mature virions were present within some of the swollen saccules (Fig. 12 *D*) virtually no virus could be detected budding at the surface of the monensin-treated WTB cells.

The most striking feature of the infected DTG 1-5-4 cells was the presence of nucleocapsid-decorated cisternae adjacent to the Golgi apparatus (Fig. 13 *A*). At times these cisternae appeared to be conformationally the same as the *trans*-Golgi saccules (Fig. 13 *B*), suggesting that they may be derived from the *trans* saccules. Although the majority of the cisternae had narrow lumina, some appeared distended and were filled with virions (Fig. 13 *C*). While some individual virus particles were seen budding at the cell surface of DTG 1-5-4 (Fig. 13 *D*), most of the virus on the surface of the mutant were present in clusters (Fig. 13 *E*). As in the WTB cells, monensin treatment of DTG 1-5-4 resulted in a swelling of the Golgi saccules (Fig. 13 *F*); however, most of the cisternae decorated with nucleocapsids appeared unaffected by the monensin. In both the untreated and monensin treated cells, the cisternal membranes appeared to be held together by numerous filamentous cross-bridges (Fig. 13 *F*, inset). These cross-bridges were also observed in monensin-treated, Sindbis-infected WTB cells.

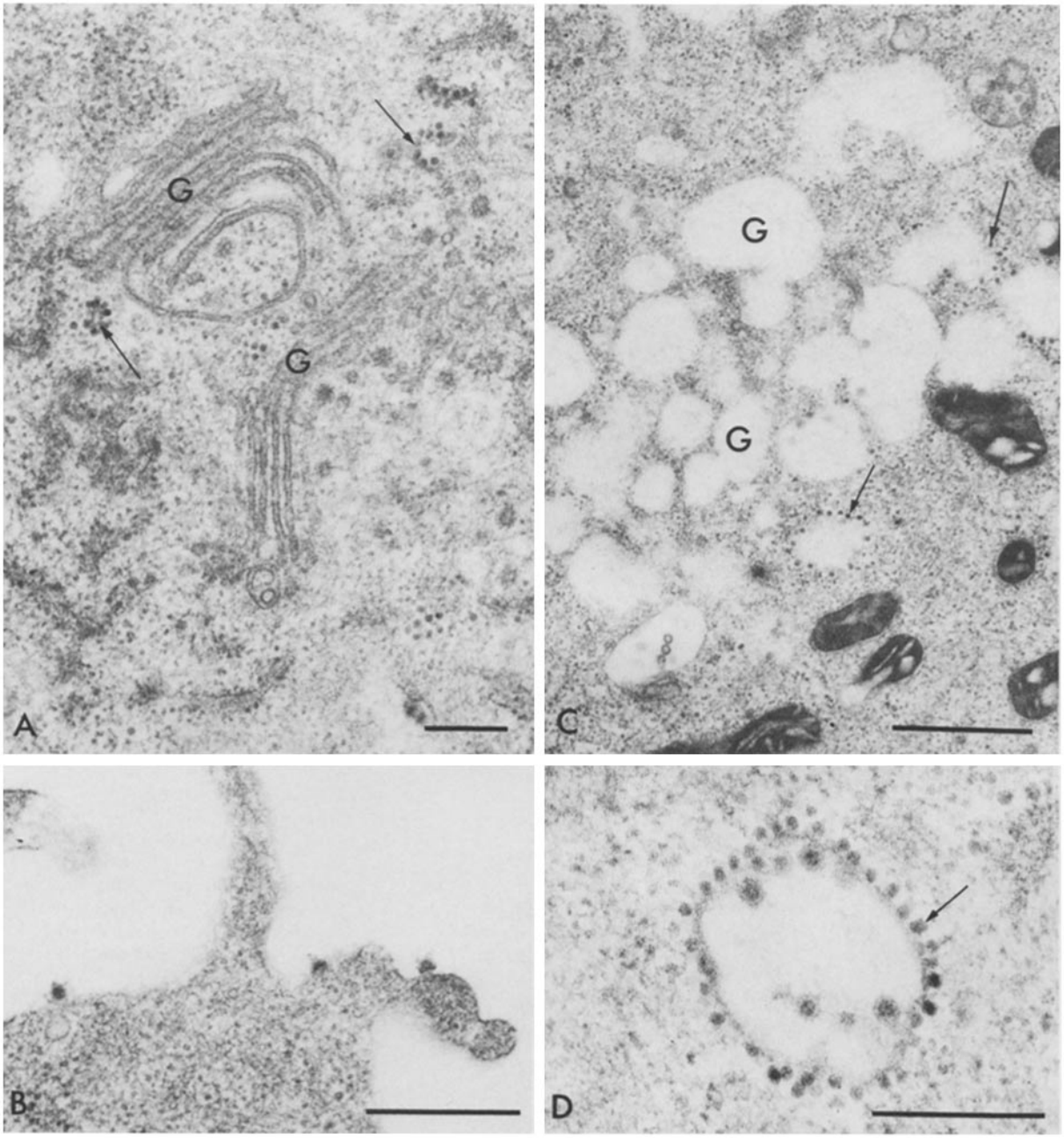

FIGURE 12 Sindbis-infected WTB cells. (*A*) In untreated cells the Golgi apparatus (*G*) consists of 5–7 saccules. Short segments of nucleocapsid-decorated cisternae are present (arrows). × 37,000. (*B*) Single virions are seen budding at the cell surface. × 50,000. (*C* and *D*) In monensin-treated cells, the Golgi saccules (*G*) are dilated and nucleocapsid (arrows) is bound to the surface of some saccules. Additionally, virions are present within an occasional saccule (*D*). (*C*) × 33,000. (*D*) × 56,000. Bars, 0.5 μm.

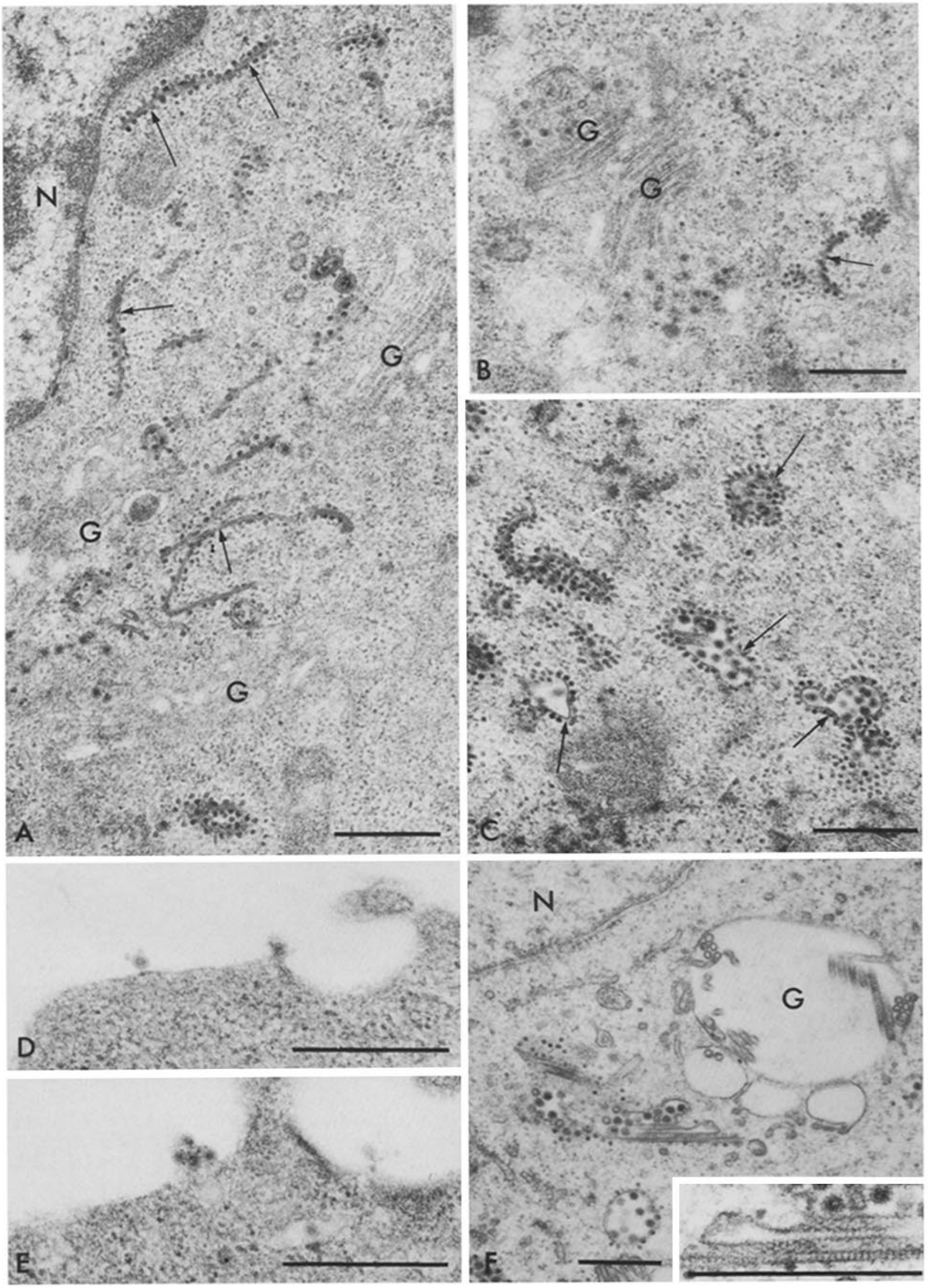

FIGURE 13 Sindbis-infected DTG 1-5-4 cells. (*A*) Numerous nucleocapsid-decorated cisternae (arrows) are present near the Golgi apparatus (*G*). *N*, nucleus. × 40,000. (*B*) These cisternae (arrow) occasionally appear morphologically similar to the *trans*-Golgi (*G*) saccules. × 37,000. (*C*) Although the cisternae are normally narrow, some appear distended and filled with virions (arrows). × 40,000. (*D* and *E*) Occasionally single virions are seen to bud from the cell surface but the majority of virus occurs in clusters. (*D*) × 58,000. (*E*) × 56,000. (*F*) In the monensin-treated cells, the Golgi saccules (*G*) are swollen, and the cisternae appear to be held together by numerous cross-bridges (*inset*). *N*, nucleus. × 32,000; (*inset*) × 86,000. Bars, 0.5 μm.

No virus was seen budding from the surface of the monensin-treated DTG 1-5-4 cells.

DISCUSSION

We have shown that two CHO cell mutants, DTF 1-5-1 and DTG 1-5-4, isolated for increased resistance to diphtheria toxin and decreased uptake via the Man 6-P receptor (25), are defective in ATP-dependent acidification of endosomes in vitro. Inhibitors of acidification block both killing by diphtheria toxin (8, 9, 57) and accumulation of ligand via the Man 6-P receptor (4); thus, the decreased endocytic activity measured in vivo with these mutants appears consistent with the decreased acidification measured in vitro. DTF 1-5-1 has been shown to be defective in release of iron from internalized diferric transferrin (28), suggesting that this mutant is deficient in endosomal acidification in vivo. Restoration of endocytosis in hybrids of DTF 1-5-1 × DTG 1-5-4-122 and in revertants of DTG 1-5-4-122 correlated with restoration of in vitro ATP-driven acidification. In vitro acidification of lysosomes from DTF 1-5-1 and DTG 1-5-4 was near normal. Thus, our results are very similar to those of Merion et al. (27), who previously demonstrated decreased ATP-dependent acidification of endosomes from RPE. 28 and RPE. 44, two CHO cell mutants selected for resistance to Pseudomonas toxin and found to be cross-resistant to diphtheria toxin and enveloped viruses (26).

DTF 1-5-1 and DTG 1-5-4 also exhibited increased resistance to Sindbis virus. Resistance could be overcome by increasing the multiplicity of infecting virions, and on infection with Sindbis at ≥20 pfu/cell the mutants synthesized viral proteins at normal levels. Surprisingly, even under these conditions both mutants released virus in reduced amounts. Examination of Sindbis-infected DTG 1-5-4 suggested that posttranslational modification of the viral glycoproteins proceeded normally only up to those steps occurring late in transit through the Golgi apparatus: proteolytic cleavage of the glycoprotein precursor pE2 to E2 was somewhat delayed and did not proceed to completion, galactosylation and sialylation of E1 and E2 were markedly reduced, transport of the glycoproteins to the cell surface was decreased, and sorting of the glycoproteins was aberrant in that some pE2 was transported to the surface of DTG 1-5-4. In addition, large numbers of viral nucleocapsids were observed bound to the cytoplasmic surfaces of cisternae very similar in appearance to Golgi saccules. These nucleocapsids were associated with viral glycoproteins, because virions were seen budding into the lumina of these cisternae. Whether a single block at one step in viral glycoprotein posttranslational modification is responsible for all the other changes observed, or whether some of these alterations occur independently of one another, is not known.

If the various phenotypic alterations observed with the mutants result from single genetic lesions, then the concomitant loss of activities associated with the Golgi complex and with the endocytic apparatus suggests that these compartments share a dependence on a common gene product. Three lines of evidence indicate that single gene defects are responsible for both decreased endocytosis and decreased Sindbis release: (*a*) spontaneous revertants showing increases in both endocytosis and virus release were obtained from DTG 1-5-4-122 at a frequency of 3×10^{-6}; (*b*) DTF 1-5-1 × DTG 1-5-4-122 hybrids were restored in both parameters; (*c*) of seven endocytosis mutants examined, all were deficient in Sindbis virus release, and the level of virus released by each mutant was proportional to the residual endocytic activity (Bateman, J. L., and A. R. Robbins, unpublished data).

What is the primary defect in mutant DTG 1-5-4? A defect in glycosylation seems unlikely in that oligosaccharides on VSV glycoprotein G and many endogenous glycoproteins were galactosylated although Sindbis glycoproteins were not; in addition, loss of galactosylation results neither in decreased Sindbis release (58), nor in resistance to diphtheria toxin (59). The marked increase in ricin sensitivity of DTG 1-5-4 also argues against a general defect in galactosylation. The mutant mimics the effects of the ionophore monensin on both the endocytic and secretory pathways: resistance to toxins (8) and decreased uptake via the Man 6-P receptor (Sahagian, G. G., manuscript submitted for publication), as well as inhibition of Sindbis glycoprotein maturation and viral release (54, 55) all have been reported for monensin-treated cells. Because of this resemblance, we suggest that some component required for ion transport in organelles of both pathways may be defective. We have shown that ATP-driven acidification of endosomes from DTG 1-5-4 is markedly reduced. While we have not yet been able to measure ion transport in Golgi fractions from CHO cells, recent studies have demonstrated that Golgi membranes from rat liver exhibit ATP-dependent H^+ transport (20, 60). Perhaps the ATPases of endosomes and Golgi share a common subunit that is altered in DTG 1-5-4; alternatively, the defect may occur in some other membrane component that is required for proton transport; for example, the activity of an anion channel has been implicated in Golgi acidification (20).

Because the mutants were isolated for endocytic defects, we tend to think of these defects as primary; however, loss of endosome acidification could result secondarily from a defect in the Golgi complex, if that defect caused lack of proper posttranslational modification and/or compartmentalization of a protein required for endosome acidification, e.g., the proton pump. This will be ascertained only on analysis of mutants with temperature-sensitive lesions.

Regardless of the relationship between the Golgi complex-associated defect and the loss of endosome acidification, the existence of a problem in the Golgi apparatus makes interpretation of the various phenotypic changes in the mutant much more complicated. The alterations reported here for the Sindbis glycoproteins could effect loss of activity of proteins (receptors and others) involved in endocytosis; for example, failure to galactosylate the receptor for modeccin in DTG 1-5-4 would explain the mutant's very high levels of resistance to that toxin, in that binding of modeccin requires a galactosylated receptor (61). Also, an acidic Golgi may itself participate in receptor-mediated endocytosis and receptor recycling. All of our mutants isolated as pleiotropically defective in endocytosis (25) exhibited decreased release of Sindbis (Bateman, J. L., and A. R. Robbins, unpublished data), and our mutants are phenotypically similar to those isolated by others (26); thus, we suspect that the presence of the Golgi defect is not unique to DTG 1-5-4.

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