Defective Transport of Sindbis Virus Glycoproteins in End4 Mutant Chinese Hamster Ovary Cells

JOHN F. PRESLEY,¹ ROCKFORD K. DRAPER,² AND DENNIS T. BROWN^{1*}

*The Cell Research Institute and Department of Microbiology, The University of Texas, Austin, Texas 78712-7640,¹ and
The Molecular and Cellular Biology Program, The University of Texas at Dallas, Richardson, Texas 75083-0688²*

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Mutant V.24.1, a temperature-sensitive derivative of Chinese hamster ovary cells, defines the End4 complementation group of mutants selected for resistance to protein toxins and has defective lysosomes at the restrictive temperature (P. A. Colbaugh, M. Stookey, and R. K. Draper, *J. Cell Biol.* 108:2211-2219, 1989). We have investigated the biosynthesis of Sindbis virus envelope glycoproteins in V.24.1 cells. When the cells were infected at the restrictive temperature, the envelope glycoproteins E1 and E2 were undetectable on the cell surface and proteolytic processing of the precursor protein pE2 to envelope protein E2 did not occur. Protein retained intracellularly was sensitive to endoglycosidase H and, by immunofluorescence localization, appeared to accumulate in the endoplasmic reticulum. We conclude that the genetic defect in V.24.1 cells impairs the transport of Sindbis virus glycoproteins, apparently at the level of export from the endoplasmic reticulum.

Vesicular trafficking inside higher eukaryotic cells is a complex and poorly understood topic. It is known that the majority of cell surface integral membrane proteins and secreted proteins follow a pathway that leads from the rough endoplasmic reticulum (RER) through the Golgi apparatus to the cell surface (1, 19). The other major vesicular trafficking pathway is the endocytic pathway, fed from clathrin-coated pits that form at the cell surface and internalize membrane and fluid (25). Most endocytosed material is either recycled to the exterior medium or delivered to lysosomes by mechanisms that are not well characterized at the molecular level.

Genetic approaches involving the production of temperature-sensitive mutants followed by phenotypic and genotypic characterization of these mutants have proven highly successful approaches for the study of complex biological systems. For example, much has been learned from studying mutants of *Saccharomyces cerevisiae* that express temperature-sensitive lesions in the secretory pathway (18). A similar genetic approach should be applicable to mammalian cells, although it is more difficult to isolate the mutants. Nevertheless, a number of temperature-sensitive mutants that express defects in either the secretory or endocytic pathway have been isolated from Chinese hamster ovary (CHO) cells (3, 10, 11, 16, 22). One such mutant, termed V.24.1, defines the End4 complementation group of mutants selected for resistance to protein toxins and has defective lysosomes at the restrictive temperature (3, 4). In the experiments presented here, we have examined the process of Sindbis virus assembly in V.24.1 cells to better characterize the defect in this mutant and to evaluate its effects on the processing and transport of model integral membrane proteins.

The Sindbis virus envelope proteins, E1 and E2, are typical integral membrane glycoproteins that are transported from the RER to the cell surface via the Golgi apparatus. These proteins are synthesized from a subgenomic RNA as a single large polypeptide that contains the capsid protein (a cytoplasmic protein that forms the virus core) at the amino terminus. The capsid protein is proteolytically removed from

the amino-terminal end while the peptide is still nascent, exposing a signal sequence that directs the remainder of the protein into the RER. During translocation, this polypeptide is cleaved (9) to give (from amino terminus to carboxy terminus) pE2 (the precursor to E2), a 6,000-Da linker peptide, and the other envelope protein, E1. PE2 is later processed to give E2 and a glycopeptide (designated E3) that is secreted into the medium. This processing event occurs predominantly after exit from the late Golgi compartments (6).

We report here that V.24.1 cells infected with Sindbis virus fail to transport viral glycoproteins to the cell surface, fail to process pE2 to E2, and appear to retain E1 and pE2 in the endoplasmic reticulum (ER), suggesting that the lesion in the mutant cells is in the secretory pathway.

MATERIALS AND METHODS

Cells and virus. Baby hamster kidney (BHK) cells were grown as described previously (24) in Eagle minimum essential medium (7) supplemented with 10% fetal calf serum, 5% tryptose phosphate broth, and 2 mM glutamine. The medium was supplemented with gentamicin (2 mM) for plaque assays. Wild-type CHO-K1 cells, from the American Type Culture collection (Rockville, Md.), were grown as described previously (4) in medium containing 5% fetal calf serum, 2 mM gentamicin, 40 µg of proline per ml, and 2 mM glutamine. Isolation of the V.24.1 cell line is described in reference 4. Continuously passaged V.24.1 cells were checked for death at the nonpermissive temperature (39.5°C) and for failure to process Sindbis envelope protein precursor pE2 at 39.5°C to determine the stability of the mutant phenotype. The mutant phenotype remained stable after 12 passages at a 1:5 dilution in the absence of selective pressure. Mutant cells were not used after 12 passages.

Stocks of the heat-resistant strain of Sindbis virus (SVHR) derived by E. R. Pfefferkorn were produced as described previously (21). Cells were infected at a multiplicity of infection of 20 as described previously (21). This multiplicity of infection is equivalent to 200 PFU per cell as determined by titration of BHK-21 cells and was needed since efficiency of plating of SVHR is 1/10 lower on monolayers of CHO cells

* Corresponding author.

than on monolayers of BHK cells. The efficiency of plating of Sindbis virus is, however, identical on V.24.1 and wild-type cells (data not shown). The multiplicity used is sufficient to infect all cells in a V.24.1 monolayer at either the permissive or nonpermissive temperature as assayed by immunofluorescence.

Cells infected at the nonpermissive temperature (39.5°C) were held at that temperature for 7 h prior to infection and during infection (1 h) to allow expression of the temperature-sensitive defect. Metabolic labeling and immunofluorescence experiments were routinely conducted 8 h postinfection. Thus, most experiments conducted at 39.5°C used cells held at the nonpermissive temperature for 16 h.

Metabolic labeling, endo H treatment, and polyacrylamide gel electrophoresis. Metabolic labeling with [³⁵S]methionine, immunoprecipitation of cell lysates, and endoglycosidase H (endo H) treatments of immune precipitates were done as previously described (13, 20). A rabbit polyclonal antibody raised against C, E1, and E2 (the viral structural proteins) was used for these immunoprecipitations. Denaturing electrophoresis in 11.8% polyacrylamide gels was done as previously described (23). Fluorography was performed by the method of Bonner and Laskey (2).

Fluorescence microscopy. Monolayers were grown in flasks mounted on slides (Nunc Inc., Naperville, Ill.) and were fixed in 3% formaldehyde for at least 12 h at 4°C prior to examination. Monolayers were washed with phosphate-buffered (PBS) and were permeabilized with PBS containing 1% Triton X-100 if internal staining was desired. Slides were then incubated in PBS containing 10% Carnation dried milk (PBS-Carnation) for 1 h. They were then incubated with a 1:100 dilution of the primary antibody in PBS-Carnation for 2 h. The primary antibody, mouse monoclonal antibody 502 (kindly supplied by Bob Johnston, Chapel Hill, N.C.), recognizes pE2 and the E2 protein of Sindbis virus. Monolayers were washed three times in PBS-Carnation and then incubated with goat anti-rabbit immunoglobulin G conjugated to rhodamine isothiocyanate (TRITC; Sigma, St. Louis, Mo.) that had previously been preadsorbed against CHO cells (1:100 in PBS-Carnation) for 1 h. Monolayers were washed again. The mounting medium was 50% glycerol–50% deionized water. Cells were examined with a Zeiss microscope and epifluorescence illumination.

RESULTS

Sindbis virus infection and growth in mutant and wild-type CHO cells. To examine virus production, monolayers of mutant and wild-type cells were infected with 20 PFU of Sindbis virus per cell as described in Materials and Methods. Medium containing progeny virus was titrated at the indicated time points in each cell line at both the permissive (34°C) and nonpermissive (39.5°C) temperatures. The resulting growth curves are shown in Fig. 1. The parental cells showed only a slight drop in titer at 39.5°C compared with 34°C. Compared with production by parental cells, virus production by V.24.1 cells at 34°C was about 10-fold reduced, but at 39.5°C production was reduced by almost 1,000-fold as assayed 24 h after infection.

Synthesis of Sindbis virus structural proteins in mutant and wild-type CHO cells. Cells that had been infected for 7 h were methionine starved for 1 h and labeled with [³⁵S]methionine continuously for 4 h as described in Materials and Methods. The monolayers were then lysed, and the virus structural proteins were immunoprecipitated and analyzed by electrophoresis in polyacrylamide gels and subsequent fluorog-

FIG. 1. Growth of Sindbis virus in mutant and parental cells at permissive and nonpermissive temperatures. Monolayers of CHO-K1 and V.24.1 cells were infected with Sindbis virus. At the indicated time points, samples of media were removed from the flasks and frozen. Virus titer was determined by plaque assay with BHK cells.

raphy (Fig. 2A). Wild-type cells showed a typical profile that included the three virus structural proteins, E1, E2, and capsid, as well as pE2, the precursor to E2. There was no significant change in the distribution of label among these proteins at the permissive or nonpermissive temperature. However, a 100,000-Da band that may correspond to an E1-pE2/E2 heterodimer or to the unglycosylated polypeptide precursor to E1 and E2 was found in both mutant and wild-type cell lysates at the permissive but not the nonpermissive temperature. The significance of the temperature sensitivity of this band is unknown.

The mutant cells differed significantly from wild type in that E2 was never detected at the nonpermissive temperature and was absent or detected in reduced amounts even at the permissive temperature. The pE2 band was, however, denser, indicating failure to process this protein to E2. Figure 2A, which shows a representative experiment, does not show E2 in the V.24.1 lanes at either temperature.

To further investigate the cleavage of pE2 to E2 at 34°C, mutant and wild-type cells were infected, and virus glycoproteins were pulse-labeled with [³⁵S]methionine for 5 min at 8 h postinfection and chased in the presence of cold methionine and cycloheximide. The extent of cleavage was monitored as a function of chase time in the absence of radiolabel. With parental cells, conversion to E2 was evident after 5 min and completed 60 min into the chase period (Fig. 3A). In contrast, pE2 cleavage in the mutant cells was extremely slow and still incomplete 2 h into the chase period (Fig. 3B).

Despite the slow processing of pE2 by V.24.1 cells at 34°C, significant (though reduced) amounts of virus were produced (Fig. 1). We previously showed that inhibition of pE2 cleavage to E2, produced by treatment with monensin in BHK cells, resulted in incorporation of pE2 into released virus (20). We therefore examined the protein composition of virus produced from V.24.1 cells to determine whether the defect in these cells resulted in the production of pE2-containing virions. Cells were infected with Sindbis virus, methionine starved for 1 h at 4 h postinfection, and labeled overnight with [³⁵S]methionine (10 μ Ci/ml). Progeny virus was purified by equilibrium centrifugation on a 20 to 35% potassium tartrate gradient, concentrated by pelleting, and dissolved in sample buffer. Electrophoresis and subsequent

FIG. 2. (A) Metabolically labeled virus structural proteins in V.24.1 and parental cells. Cells were metabolically labeled for 4 h with [35 S]methionine starting at 8 h postinfection as described in the text, and Sindbis virus proteins were immunoprecipitated. Monolayers maintained at the nonpermissive temperature were held at that temperature for 7 h prior to infection. SV, Purified Sindbis virus marker. (B) E1 and E2 in mature virus produced by infected V.24.1 cells at the permissive temperature. Lanes: BHK, E1 and E2 in Sindbis virus grown in BHK cells; V.24.1, E1 and E2 in virus grown in V.24.1.

fluorography showed normal amounts of E1 and E2 in virions (Fig. 2B). No pE2 was detected even with prolonged exposure of the gel. This result indicates that the mechanism by which the pE2-to-E2 cleavage is inhibited in V.24.1 cells differs from the mechanism by which processing is inhibited in monesin-treated cells.

In summary, virus production in V.24.1 cells is mildly reduced at 34°C, and this reduction is correlated with a slow cleavage of pE2 to E2. Virus produced in V.24.1 cells at 34°C contains normal E2 and E1, indicating that pE2 is not available for incorporation into virus. At 39.5°C, both virus production and cleavage of pE2 to E2 are strongly inhibited.

Processing of virus glycoproteins in mutant and wild-type

cells. Sensitivity of oligosaccharide groups of membrane glycoproteins to the enzyme endo H was used as an assay for measuring the extent of transport and processing of Sindbis virus proteins in the mutant and wild-type cells. Endo H removes high-mannose N-linked oligosaccharides added in the RER but cannot cleave these same oligosaccharides after they have been processed to complex forms by enzymes in the medial Golgi stacks (12, 13). Thus, endo H sensitivity or resistance reveals whether particular proteins have been transported through the medial Golgi stacks, assuming that the protein is in a conformation in which oligosaccharide groups are accessible to the modifying enzymes.

Mutant and parental cells were infected with virus 8 h after a shift to the restrictive or permissive temperature. At 7 h postinfection, monolayers were starved for methionine for 1 h. They were subsequently pulse-labeled (at 8 h postinfection) with [35 S]methionine for 15 min and chased for 45 min in the presence of unlabeled methionine and cycloheximide. The cells were lysed to terminate the chase, and virus proteins were immunoprecipitated as described in Materials and Methods. Immunoprecipitated proteins were treated or mock treated with endo H, dissolved in sample buffer, and electrophoresed. Protein bands were visualized by fluorography. Wild-type cells showed a mixture of endo H-resistant and -sensitive envelope virus proteins at both permissive and nonpermissive temperatures, with endo H-resistant forms of the envelope proteins predominating (Fig. 4a). In general, pE2 was endo H sensitive and E2 was endo H resistant. E1 was present as both endo H-sensitive and -resistant species (Fig. 4a). These results are similar to those obtained by Knipfer and Brown in a BHK cell line at 37°C (13). In V.24.1 cells, by contrast, the virus proteins failed to become endo H resistant at 39.5°C (Fig. 4a). Virus proteins remained endo H sensitive after 3 h of chase in V.24.1 cells at the nonpermissive temperature (data not shown). At 34°C, the bulk of the envelope proteins (E1 and pE2 plus E1) were endo H sensitive, with a very faint band of endo H-resistant E2 detected (Fig. 4a).

These results can be interpreted in two ways. Either Sindbis virus envelope proteins are not transported through the Golgi apparatus in V.24.1 cells during the time of the chase (1 h) or they are being transported without proper modification of N-linked oligosaccharide groups on the proteins. If the lesion in the cells blocks modification, then the

FIG. 3. Rate of pE2 cleavage in infected V.24.1 and wild-type cells at 34°C. Cells were pulsed with [35 S]methionine for 5 min and chased for the times indicated except that cells were pulsed for 15 min for chase times of 120 and 180 min. (A) CHO-K1 cells; (B) V.24.1 cells.

FIG. 4. (a) Endo H sensitivity of intracellular virus proteins. Monolayers were labeled at 8 h postinfection as described in the text. Monolayers were pulsed for 15 min with [35 S]methionine and chased for 45 min in the presence of cycloheximide. Samples were then treated with endo H (+) or mock treated (-). Lanes: A, Sindbis virus grown in BHK cells (negative control); B, virus proteins from a BHK monolayer pulsed for 5 min with no chase (positive control); C, CHO-K1 cells maintained at 39.5°C; D, V.24.1 cells maintained at 39.5°C; E, CHO-K1 cells maintained at 34°C; F, V.24.1 cells maintained at 34°C. (b) Endo H sensitivity of proteins in mature virus. Lanes: BHK, virus grown in BHK monolayers metabolically labeled with [35 S]methionine; V.24.1, virus grown in V.24.1 monolayers metabolically labeled with [35 S]methionine at 34°C. All monolayers were labeled beginning at 8 h postinfection.

envelope proteins in that population of virus that is released from the mutant should not be endo H resistant. To check this, radiolabeled envelope proteins were prepared from purified virus grown on V.24.1 cells at 34°C and treated with endo H. Only endo H-resistant protein was present despite the inefficient conversion of envelope proteins to endo H-resistant forms in these cells. This finding implies that the modification enzymes in the medial Golgi stacks are functional and indicates that endo H resistance is a valid indicator of protein transport in these cells.

Immunofluorescent localization of virus proteins in mutant and wild-type cells. Immunofluorescence was used to determine the distribution of virus proteins within virus-infected cells and as an assay for transport of virus protein to the cell surface. Parental and mutant cells were infected with Sindbis virus at both permissive and nonpermissive temperatures. At 8 h postinfection, monolayers were fixed in 3% formaldehyde. The cells were permeabilized with 1% Triton X-100 for 10 min prior to staining if intracellular staining was desired. Cells were stained with a primary mouse monoclonal antibody against the E2 protein and a goat anti-mouse secondary antibody conjugated to rhodamine. Staining of nonpermeabilized cells, which detects envelope proteins E1 and E2 on the surface of infected cells, showed substantial protein on the surface of parental cells at 34°C and a visible but weaker staining on the surface of V.24.1 cells (data not shown). At 39.5°C, the wild-type cells were intensely stained, but V.24.1 cells were not distinguishable from uninfected controls, indicating that virus proteins were not reaching the cell surface (Fig. 5).

Staining of permeabilized virus-infected V.24.1 cells at the nonpermissive temperature showed a striking perinuclear reticulated staining pattern (Fig. 6B). This pattern has been reported for RER-associated proteins and is distinct from the pattern observed in the wild-type cells at 39.5°C (Fig. 6A). It is also distinct from the pattern seen when V.24.1 cells were stained with the Golgi apparatus-specific marker wheat germ agglutinin (data not shown) or when wild-type cells were infected with the Sindbis virus conditional lethal mutant *ts23*

(13), which accumulates the envelope proteins in the Golgi apparatus (data not shown). The results with V.24.1 cells at the restrictive temperature probably reflect accumulation of virus envelope proteins in the ER. Interestingly, the heaviest staining was consistently concentrated near the nucleus and in the nuclear envelope. It is possible that virus protein was concentrated in the transitional elements of the ER or that cytoskeletal disorganization resulting from the virus infection had resulted in structural collapse of portions of the ER to the perinuclear region. This staining pattern does not represent failure of the pE2 protein in other cellular locations to express the epitope recognized by the monoclonal antibody since an identical staining pattern was obtained after staining of V.24.1 cells with a polyclonal antibody made against all of the Sindbis virus structural proteins (data not shown).

DISCUSSION

V.24.1 cells were derived from CHO-K1 cells by using a protocol designed to isolate temperature-sensitive mutants resistant to protein toxins (3). At the restrictive temperature, dense lysosomes in the mutant cells were almost devoid of acidification activity and the activities of three lysosomal enzymes tested were reduced (4). One explanation for the phenotype of the mutant could be the existence of a defect in the RER-to-Golgi aspect of the secretory transport pathway involved in stocking the lysosomal compartment with enzymes and membrane proteins. Thus, we examined the maturation of Sindbis virus integral membrane proteins pE2/E2 and E1, using the virus proteins as probes to determine whether the secretory pathway in V.24.1 cells was functioning properly. Maturation of Sindbis virus glycoproteins involves modifications in oligosaccharide groups, changes in protein conformation, and the proteolytic cleavage of pE2, providing multiple points at which proper functioning of the protein secretory pathway can be assessed.

Sindbis envelope glycoproteins did not reach the surface

FIG. 5. Expression of virus proteins on the cell surface. (A) Wild-type cells maintained at 39.5°C from 7 h prior to infection until the monolayer was fixed in 3% formaldehyde (8 h postinfection). The cells were then stained with an anti-E2 monoclonal primary antibody and a TRITC-conjugated goat anti-rabbit secondary antibody. (B) V.24.1 cells infected and stained as were parental cells. (C) Phase contrast of panel A. (D) Phase contrast of panel B.

FIG. 6. Immunofluorescent localization of virus proteins in detergent-permeabilized cells. Cells were held at 39.5°C from 7 h prior to infection with Sindbis virus to 8 h after infection. They were then fixed in 3% formaldehyde, permeabilized with Triton X-100, and stained as described in Materials and Methods. Panels: A, Wild-type cells; B, V.24.1 cells; C, phase contrast of panel A; D, phase contrast of panel B.

of V.24.1 cells infected at the restrictive temperature, and they were retained intracellularly in an endo H-sensitive form, suggesting that they had not reached the medial Golgi saccules. Proteolytic cleavage of pE2, believed to occur primarily after exit from the Golgi apparatus, was also strongly inhibited. Moreover, proteins accumulated intracellularly in a perinuclear reticulated pattern, suggesting that they were in the ER. The data strongly suggest that the lesion in the mutant cells impairs the secretory pathway, probably at the level of export from the ER.

V.24.1 cells can grow indefinitely at 34°C (3, 4). It was curious, therefore, that virus production and the maturation of virus glycoproteins was so strongly affected even at the permissive temperature. It would seem unlikely that a cell expressing such an impairment of an intracellular transport pathway would be viable. This observation suggests that the lesion may be exacerbated at the permissive temperature by the process of viral infection. We previously noted that protein synthesis was necessary for V.24.1 cells at 39.5°C to recover upon the shift back to 34°C (4), implying that the mutated gene product responsible for the lesion was labile and had to be synthesized anew for recovery to occur. It is possible that the defective gene product is more labile than normal even at 34°C and that when protein synthesis is inhibited by viral infection, not enough of the defective gene product accumulates to support normal function. Thus, it is conceivable that viral infection at the permissive temperature could enhance the mutant phenotype simply by inhibiting host protein synthesis.

The lesion in V.24.1 cells causing aberrant trafficking of Sindbis virus glycoproteins could result from a defect in N-linked glycosylation that renders the proteins incompetent for transport. Hearing and co-workers (10, 11) described such a lesion in CHO cells caused by the addition of truncated N-linked oligosaccharides to secreted proteins. This is an unlikely explanation for the lesion in V.24.1 cells, however, because we found in preliminary experiments that Sindbis virus proteins that did not contain N-linked oligosaccharides as a result of the presence of tunicamycin were retained intracellularly by V.24.1 cells, although appreciable amounts of protein were detected on the cell surface in tunicamycin treated wild-type CHO cells at 39.5°C (data not shown). We have shown previously that tunicamycin inhibition of glycosylation does not completely block transport of Sindbis virus glycoproteins to the cell surface in BHK cells (15, 23). We have found that there is also a strong block in the transport of soluble secreted glycoproteins and of the transferrin receptor in V.24.1 cells at the nonpermissive temperature (26). Thus, the lesion in the mutant cells is not restricted to virus glycoproteins.

The available evidence suggests that the lesion in the mutant cells might impair some fundamental step involved in protein export from the ER. The folding of proteins within the ER to attain a proper tertiary structure is important for export (5, 8, 14). The heavy-chain-binding protein is believed to assist nascent proteins in folding (8), and a lesion in this or some other protein that affects folding could underlie the defect in V.24.1 cells. Alternatively, there could be a defect in forming vesicles that carry cargo from the ER to the Golgi or in the fusion of such vesicles with the Golgi, similar to what has been found for the yeast *sec18* mutant, (17, 18) in which the *N*-ethylmaleimide-sensitive factor biochemically characterized by Wilson and co-workers (27) is affected. Work is currently under way to investigate these possibilities by using *in vivo* and *in vitro* approaches.

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