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A Mutant CHO-K1 Strain with Resistance to *Pseudomonas* Exotoxin A and Alphaviruses Fails To Cleave Sindbis Virus Glycoprotein PE2

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RPE.40, a mutant CHO-K1 strain selected for resistance to *Pseudomonas* exotoxin A, is defective in the production of infectious alphaviruses, although viruses are taken in and processed normally (J. M. Moehring and T. J. Moehring, Infect. Immun. 41:998–1009, 1983). To determine the cause of this defect, the synthesis of Sindbis virus proteins was examined. RPE.40 cells produced and glycosylated structural glycoprotein precursors PE2 and immature E1 normally. Mature E1 was formed, but PE2 was not cleaved to E2 and E3. PE2 instead was modified to a higher-molecular-weight form (PE2′) in which the high-mannose oligosaccharides were processed to the complex form without proteolytic cleavage. The data suggest that the cleavage which produces E2 occurs within the trans-Golgi or in post-Golgi elements and is closely associated with the addition of sialic acid residues to the asparagine-linked oligosaccharides. RPE.40 cells make and release noninfectious Sindbis virions that contain PE2′ and no detectable E2. These virions can be converted to an infectious form by treatment with trypsin. A defect in an intracellular endopeptidase activity in RPE.40 cells is postulated. Comparison of two Sindbis virus strains showed that the requirement for E2 in the virion to ensure infectivity is strain specific.

We previously reported the isolation and preliminary characterization of a mutant Chinese hamster ovary (CHO) cell strain which was resistant to *Pseudomonas* exotoxin A. This strain, RPE.40, was selected by a single exposure of recently cloned CHO-K1 cells to *Pseudomonas* toxin (21). RPE.40 cells also proved defective in production of infectious Sindbis virus (SV), Semliki Forest virus (SFV), and a third alphavirus, Chikungunya virus (CV). *Pseudomonas* toxin, like diphtheria toxin, catalyzes the transfer of the ADP-ribose moiety of NAD^+ to elongation factor 2, causing its inactivation and cell death (13). RPE.40 cells showed no alteration in sensitivity to diphtheria toxin or to several toxic lectins. In addition, RPE.40 cells were not altered in their ability to replicate vesicular stomatitis virus. To date, no other mutant mammalian cells with this phenotype have been described.

RPE.40 cells contain a normal complement of toxin-sensitive elongation factor 2, suggesting that their genetic lesion affects entry, activation, or intracellular transport of *Pseudomonas* toxin. SV-infected RPE.40 cells produce normal amounts of viral RNA but a substantially reduced number of infectious virions, suggesting that these cells possess a mutation affecting a step in the late stage of SV replication. We hypothesized that RPE.40 cells might be defective in an enzyme required both for the processing of alphavirus structural proteins and for the intracellular activation of *Pseudomonas* toxin (21). Alternatively, the enzyme could be required for a step in the biosynthesis of the *Pseudomonas* toxin receptor. Since the replication of SV is well characterized (31), a detailed examination of the processing of the SV structural proteins was undertaken to provide insight into the mechanism responsible for the phenotype of the RPE.40 cell.

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SV is an enveloped RNA virus belonging to the genus *Alphavirus* of the family *Togaviridae*. The virion consists of single-stranded RNA complexed with a basic capsid protein (C-protein), surrounded by a lipoprotein envelope into which the two major viral glycoproteins, E1 and E2, are inserted (33). The SV structural proteins are produced as a polyprotein translated from a subgenomic 26S RNA. Mature viral glycoprotein E2 is derived by proteolysis of a larger precursor, designated PE2 (32). After the C-protein is released by an autoprotease activity, PE2 and E1 are cotranslationally inserted into the membrane of the endoplasmic reticulum and then separated by two cleavage events (34). PE2 and E1 are glycosylated by the en bloc transfer of a simple carbohydrate chain ($Glc_3Man_9GlcNAc_2$) from a dolichol phosphate intermediate to asparagine residues present in the sequence Asp-X-Ser/Thr (17). Three glycosylation sites are located on PE2 and two sites are found on E1 (10, 25). As the glycoproteins are transported through the Golgi, the carbohydrate side chains of at least one site on each protein undergo a series of modifications by host cell enzymes which convert the oligosaccharides from the high-mannose form to a complex form (11, 12, 19). In the course of this conversion, an immature form of E1, lacking sialic acid residues, can be detected (2). PE2 is cleaved to yield mature E2 and a small glycoprotein, E3, which is released into the medium (35).

Prior to recent reports which showed that infectious SV may contain uncleaved PE2 (23, 27), it was believed that cleavage of PE2 was an essential step in the maturation of infectious SV. It has been proposed that this cleavage takes place at the plasma membrane, just prior to virion release (1, 29), or that this event is catalyzed by a cellular protease located within the trans-Golgi cisternae or in post-Golgi elements (2, 9, 25). The recent studies of Knipfer and Brown (16) indicate that cleavage of PE2 may occur independently of cellular protein transport in BHK-21 cells. Mature E1 and E2 glycoproteins are transported to the cell membrane,

where they associate with the nucleocapsid prior to budding of the virion from the cell.

In this article, we report that RPE.40 cells fail to produce normal titers of infectious SV due to a defect in their ability to cleave PE2 to E2, which is apparently required for infectivity by SV strain AR339 but not by strain S.A.AR86.

MATERIALS AND METHODS

Cells. CHO-K1 Chinese hamster ovary cells were obtained from the American Type Culture Collection (Rockville, Md.). The isolation and preliminary characterization of mutant strain RPE.40 have been described previously (21). Cells were cultured in Ham's nutrient mixture F-12 containing 5% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO_2 in air.

Viruses and antisera. The AR339 strain of SV was originally obtained from B. Forsyth (University of Vermont, Burlington). SV strains S.A.AR86 and S12, the cleavage-defective mutant isolated from it (27), were received from the laboratory of R. E. Johnston (University of North Carolina, Chapel Hill). Rabbit antibodies specific for Sindbis virus glycoproteins E1 and E2 were provided by A. Schmaljohn (USAMRIID, Fredrick, Md.). SFV and CV were obtained from the American Type Culture Collection. All viral stocks were prepared in CHO-K1 cells. Titers of infectious virus were determined by plaque assay with an overlay of F-12 growth medium with 30 mM $MgCl_2$ and 0.8% agarose. Plaques were visualized by using a second agarose overlay containing 0.007% neutral red.

Metabolic labeling. Cells were seeded in 35-mm culture dishes at a density of 7.5×10^4 cells per dish in 3 ml of medium and incubated for 36 h. Cells were infected with virus at 5 to 10 PFU/cell in 0.5 ml of medium. After adsorption for 1 h at 37°C, cultures were washed to remove unadsorbed virus, and 2 ml of F-12 growth medium containing dactinomycin (4 μg/ml) was added. Prior to labeling, cultures were washed twice and incubated for 15 min in minimal essential medium (MEM) lacking methionine or containing 1/10 the normal concentration of glucose when labeling with tritiated sugars (6). Each medium contained 5% dialyzed FBS and dactinomycin (4 μg/ml). To label with [^{35}S]methionine, cultures were incubated for 10 min in methionine-free MEM containing 100 μCi of [^{35}S]methionine (Du Pont, Boston, Mass.) or Tran^{35}S-label (ICN, Irvine, Calif.) per ml. Cultures were pulse-labeled with tritiated sugars at 37°C in reduced-glucose MEM containing 50 μCi of either [^{3}H]mannose, [^{3}H]fucose, or [^{3}H]galactose per ml. To chase radioactivity, the labeling medium was removed, and cells were washed once and then incubated in F-12 growth medium. At the end of the chase period, cells were washed twice in phosphate-buffered saline (pH 7.4) and once in lysing buffer (100 mM Tris [pH 7.4], 100 mM KCl, 1.5 mM magnesium acetate). Cells were solubilized in lysing buffer containing 1% sodium dodecyl sulfate (SDS) and 1% Nonidet P-40. Extracts were passed through a 26-gauge needle and frozen at −70°C.

To prepare radiolabeled virus, virus-infected cultures pulse-labeled with [^{35}S]methionine as described above were incubated for an additional 4 h at 37°C in growth medium containing 25 μCi of [^{35}S]methionine per ml and dactinomycin (4 μg/ml). Culture supernatant was then collected and centrifuged at 2,000 × *g* for 15 min. Viruses were pelleted at 100,000 × *g*, purified on a 25 to 50% sucrose gradient in TNE buffer (0.05 M Tris [pH 7.5], 0.1 M NaCl, 0.001 M EDTA), and stored at −70°C.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the procedure of Laemmli (18), with 10% polyacrylamide resolving gels and 3% polyacrylamide stacking gels. Gels were dried, and autoradiographs were prepared with Kodak X-Omat XAR-5 film. Gels containing ^{3}H-labeled proteins were treated for fluorographic detection of radioactivity by the procedure of Chamberlain (3).

Immunoblotting. Proteins on SDS-PAGE gels were transferred to nitrocellulose in a Hoefer Transphor electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) at 15 V for 12 h. Transblots were washed three times in TS buffer (20 mM Tris [pH 7.5], 0.5 M NaCl) and soaked in TS buffer containing 3% gelatin for 1 h. Blots were washed with TS buffer between each of the subsequent incubations. All incubations were performed at room temperature. Rabbit antibodies to Sindbis virus glycoproteins E1 and E2 were diluted in TS buffer, added to the transblots, and incubated overnight. Blots were soaked for 30 min in TS buffer containing 1% gelatin or 2% skim milk powder and then incubated for 4 h in TS buffer containing 1% gelatin and biotinylated goat anti-rabbit immunoglobulin G (IgG) (Vector Labs, Burlingame, Calif.). Blots were then incubated for 1.5 h in TS buffer containing 1% gelatin and avidin-conjugated peroxidase (Cappel, Malvern, Pa.). Immunoreactive bands were visualized by the addition of a DAB substrate solution (100 mM Tris [pH 7.5], 0.08% diaminobenzidine tetrahydrochloride, 0.04% $NiCl_2$, 0.01% H_2O_2).

Enzymatic digestion of glycoproteins. Sindbis virus glycoproteins were analyzed after treatment with endo-β-*N*-acetylglucosaminidase H (endo H; ICN, Lisle, Ill.), endo-β-*N*-acetyl-glucosaminidase F (endo F; Calbiochem, San Diego, Calif.), or neuraminidase (Calbiochem). Extracts of cells infected with SV were diluted 1:10 with either endo H buffer (200 mM sodium citrate, pH 5.5), endo F buffer (100 mM sodium phosphate, pH 6.0, 50 mM EDTA, 1% 2-mercaptoethanol), or neuraminidase buffer (100 mM sodium acetate, pH 5.0). Samples were boiled for 2 min and cooled to 37°C. Samples then were incubated for 20 h with or without endo H (0.01 U/ml), endo F (0.6 U/ml), or neuraminidase (0.3 U/ml). After enzyme treatment, proteins were precipitated with 10% trichloroacetic acid at 4°C and pelleted in a Beckman microfuge. Pellets were washed in ethanol-ether (1:1), repelleted, and washed in distilled H_2O. The proteins then were dissolved in SDS-PAGE sample buffer by overnight incubation at room temperature.

Trypsin activation of alphaviruses. Preparations of alphaviruses grown in either CHO-K1 or RPE.40 cells were diluted in calcium- and magnesium-free phosphate-buffered saline (pH 7.4) containing 0.02% EDTA and incubated in the presence or absence of trypsin (10 μg/ml) for 30 min at 37°C. Samples were then diluted into ice-cold Earle's balanced salts solution containing 2% FBS, and plaque assays were performed. [^{35}S]methionine-labeled SV prepared in either CHO-K1 or RPE.40 cells was treated with trypsin as above, diluted into SDS-PAGE sample buffer, and analyzed by SDS-PAGE.

RESULTS

To determine the cause of reduced titers of infectious SV from RPE.40 cells, we first examined the formation and processing of viral proteins in both wild-type CHO-K1 and mutant RPE.40 cells by pulse-chase analysis. SV-infected CHO-K1 and RPE.40 cells were pulse-labeled with Tran^{35}S-label for 10 min at 37°C and then incubated for up to 90 min

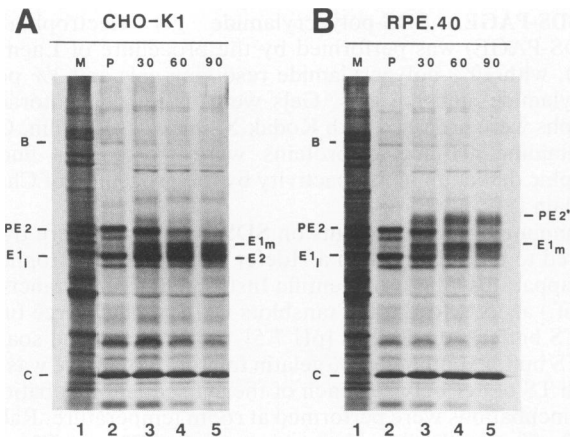

FIG. 1. SDS-PAGE analysis of CHO-K1 (A) and RPE.40 (B) cell extracts prepared from mock-infected or SV-infected cultures. Cells were infected with SV at 10 to 20 PFU/cell and incubated at 37°C in Ham's F-12 medium containing dactinomycin (4 μg/ml). At 8 h postinfection, cells were pulsed for 10 min with methionine-free MEM containing 100 μCi of Trans^{35}S-label per ml. Radioactivity was then chased by further incubation at 37°C in Ham's F-12 medium containing dactinomycin for up to 90 min. Lane 1, Mock-infected cells; lane 2, 10-min pulse; lane 3, 30-min chase; lane 4, 60-min chase; lane 5, 90-min chase.

in the absence of the label. Cell extracts were prepared and analyzed by SDS-PAGE. An autoradiogram from a typical experiment is shown in Fig. 1. After a 10-min pulse, no differences were detected in cell extracts prepared from mock-infected CHO-K1 (Fig. 1A, lane 1) and RPE.40 cells (Fig. 1B, lane 1). In both wild-type and mutant SV-infected cells, SV proteins B (97 kDa), PE2 (62 kDa), immature E1 ($E1_i$) (52 kDa), and C (30 kDa) were produced in comparable amounts (lane 2). This demonstrated that RPE.40 cells are not altered in their ability to produce the SV structural precursor proteins.

When radioactivity in SV-infected CHO-K1 cells was chased at 37°C, the conversion of the structural precursors PE2 and immature E1 (identified as $E1_i$) to E2 and the mature E1 (identified as $E1_m$) was observed. Likewise, in RPE.40 cells the appearance of mature E1 and corresponding decrease in labeled immature E1 showed that RPE.40 cells were not altered in their ability to produce the mature E1 glycoprotein. However, mature E2 glycoprotein, which migrated only slightly ahead of immature E1 in these gels, did not accumulate in these cells as it did in CHO-K1. Instead, RPE.40 cells produced and accumulated a viral protein of 65 to 67 kDa which we designated PE2′. (Positive identification of E1- and E2-related proteins by specific antibodies is shown in Fig. 2 and 3.) PE2′ was present in SV-infected RPE.40 cells chased for as short a time as 15 min (data not shown). The appearance of PE2′ and corresponding decrease in the amount of labeled PE2 suggested that PE2′ was formed by posttranslational processing of PE2. When cultures were pulse-chased starting at 8 h postinfection, a protein having the same mobility as PE2′ was also observed to form transiently in SV-infected CHO-K1 cells (Fig. 1A, lane 3). However, PE2′ was not present in CHO-K1 cultures pulse-chased at 5 h postinfection (data not shown).

At 15°C, viral glycoproteins exit the endoplasmic reticulum but accumulate in pre-Golgi vacuoles (28), whereas at 30°C the proteins pass through the Golgi and reach the

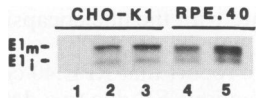

FIG. 2. Identification of SV E1 glycoproteins by Western immunoblot analysis. SV-infected CHO-K1 and RPE.40 cell extracts were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, incubated with SV E1 antiserum, and immunostained as described in Materials and Methods. Lane 1, Mock-infected CHO-K1 cells; lane 2, SV-infected CHO-K1 cells at 5 h postinfection; lane 3, SV-infected CHO-K1 cells at 8 h postinfection; lane 4, SV-infected RPE.40 cells at 5 h postinfection; lane 5, SV-infected RPE.40 cells at 8 h postinfection.

plasma membrane. To examine the processing of PE2 to PE2′ in RPE.40 cells, SV-infected cells were pulsed at 37°C, and radioactivity was chased at either 37, 30, or 15°C. At 15°C, labeled PE2 was not chased and no PE2′ was produced even after a 2-h incubation. Likewise, the processing of immature E1 to mature E1 did not occur. However, when radioactivity was chased at 30°C, both PE2′ and mature E1 were produced at rates similar to those observed at 37°C. These results suggested that, like the processing of immature E1 to mature E1, the posttranslational conversion of PE2 to PE2′ required processing of PE2 in the Golgi (data not shown).

SV structural glycoproteins were positively identified by Western immunoblot analysis of SV-infected CHO-K1 and RPE.40 cells with E1- or E2-specific antiserum. Western blot analysis of SV-infected cell extracts with anti-E1 antibody (Fig. 2) demonstrated that both the immature ($E1_i$) and mature ($E1_m$) forms of the E1 glycoprotein were present in CHO-K1 cells. Comparable amounts of these two glycoproteins were also present in mutant RPE.40 cells. Neuraminidase treatment confirmed that our immature E1 corresponds to the early form of E1, lacking sialic acid residues, described by Bonatti and Cancedda (2) (see Fig. 5).

Immunoblot analysis of extracts of SV-infected CHO-K1 cells with anti-E2 antibody (Fig. 3) demonstrated the presence of mature glycoprotein E2 and its precursor PE2. Again, no mature E2 glycoprotein could be demonstrated in RPE.40 cells. PE2 and PE2′ were the only viral proteins recognized in these cells by the E2 antiserum. Virions released from both cell strains likewise contained mature E1, but only those from CHO-K1 contained mature E2. Those from RPE.40 had only PE2′ (see Fig. 6). These data suggested that RPE.40 cells failed to produce a normal titer of infectious SV because they were unable to properly process PE2 to the mature E2 glycoprotein.

Since RPE.40 cells are also resistant to SFV and CV, the

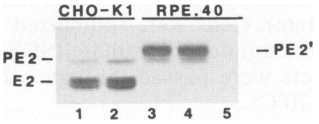

FIG. 3. Identification of SV E2 glycoproteins by Western immunoblot analysis. SV-infected CHO-K1 and RPE.40 cell extracts were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, incubated with SV E2 antiserum, and immunostained as described in Materials and Methods. Lane 1, SV-infected CHO-K1 cells at 5 h postinfection; lane 2, SV-infected CHO-K1 cells at 8 h postinfection; lane 3, SV-infected RPE.40 cells at 5 h postinfection; lane 4, SV-infected RPE.40 cells at 8 h postinfection; lane 5, mock-infected RPE.40 cells.

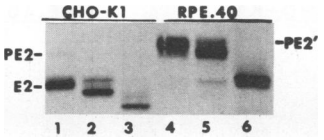

FIG. 4. Western immunoblot analysis of SV glycoproteins before and after treatment with endo H or endo F. Cell extracts from SV-infected CHO-K1 (lanes 1 to 3) or RPE.40 cells (lanes 4 to 6) were digested with 10 mU of endo H per ml (lanes 2 and 5), 600 mU of endo F per ml (lanes 3 and 6), or mock digested (lanes 1 and 4). Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. Nitrocellulose blots were incubated overnight in the presence of SV E2 polyclonal antiserum and immunostained as described in Materials and Methods.

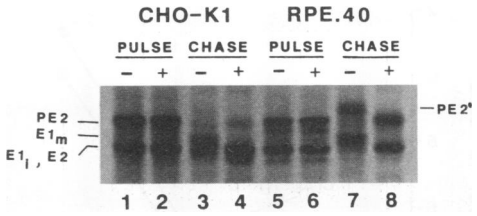

FIG. 5. SDS-PAGE analysis of SV glycoproteins from CHO-K1 (lanes 1 to 4) and RPE.40 (lanes 5 to 8) cells following neuraminidase treatment. ^{35}S-labeled SV-infected cell extracts (10-min pulse or 10-min pulse with a 90-min chase) were incubated for 24 h at 37°C with (+) or without (−) neuraminidase (0.3 U/ml). Proteins were precipitated with 10% trichloroactic acid, washed in ethanol-ether (1:1), and dissolved in SDS-PAGE sample buffer.

glycoproteins of these viruses were examined following pulse-chase labeling of infected cells with [^{35}S]methionine. The results indicated that RPE.40 cells infected with each of these alphaviruses also accumulated a PE2′-like material (data not shown). All other viral proteins appeared to be produced normally in RPE.40 cells infected with SFV or CV.

Metabolic labeling of SV-infected RPE.40 cells showed that they were not defective in oligosaccharide processing, nor did they hyperglycosylate glycoproteins, defects which have been reported for certain alphavirus-resistant L cells (8). [^{3}H]mannose was apparent in the normal viral glycoproteins and in PE2′. [^{3}H]galactose and [^{3}H]fucose were incorporated, primarily into mature glycoproteins E1, E2, and PE2′ (data not shown). Minor and transient amounts of a PE2′-like protein were noted in CHO-K1 cells. These findings demonstrated that PE2′ is a form of PE2 in which high-mannose oligosaccharides have been processed to the complex form.

To study further the processing of the PE2 glycoprotein, extracts of cells infected with SV were treated with endo H or endo F enzymes, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting with anti-E2 antiserum. Endo H cleaves high-mannose-type oligosaccharides from glycoproteins, whereas endo F removes both high-mannose and complex oligosaccharides. The results are shown in Fig. 4. The electrophoretic mobility of SV glycoprotein PE2 from CHO-K1 cells was significantly increased by endo H treatment, whereas the mobility of E2 showed only a slight increase (lane 2). The electrophoretic mobility of E2 was, however, significantly increased upon digestion with endo F (lane 3). These data are consistent with published reports showing that PE2 contains high-mannose oligosaccharides (2, 9), while E2 contains complex oligosaccharides in combination with oligosaccharides which are not accessible to enzymatic alteration and therefore remain in the high-mannose form (11). When the PE2 glycoprotein produced in RPE.40 cells was treated with endo H, its mobility was increased, as was that of endo H-treated PE2 from CHO-K1 cells. Treatment with endo H had only a slight effect on the mobility of PE2′ produced in RPE.40 cells (lane 5). The mobility of PE2′ was, however, significantly increased following endo F treatment (lane 6). These results provided additional evidence that PE2′ is an altered form of PE2 in which the majority of the high-mannose oligosaccharide side chains have been processed to the complex form.

^{35}S-labeled SV glycoproteins were also examined for the presence of sialic acid by digestion with neuraminidase. The resulting products were analyzed by SDS-PAGE (Fig. 5). Neither PE2 nor immature E1, in extracts of CHO-K1 and RPE.40 cells (lanes 1 and 5), were altered by neuraminidase treatment (lanes 2 and 6). However, the electrophoretic mobilities of the mature glycoproteins E1 and E2 present in extracts of CHO-K1 cells (lane 3) increased following neuraminidase treatment (lane 4), demonstrating that sialic acid residues were present on these glycoproteins. Likewise, the PE2′ and mature E1 glycoproteins present in SV-infected RPE.40 cells (lane 7) were also sensitive to neuraminidase. These results confirm that the oligosaccharides of PE2′ have been processed to the complex form and possess terminal sialic acid residues.

To examine the nature of the SV particles produced in CHO-K1 and RPE.40 cells, culture fluid from ^{35}S-labeled infected cells was collected 12 h postinfection. Radiolabeled virions were gradient purified and analyzed by SDS-PAGE. The results demonstrated that SV produced in RPE.40 cells contained the capsid (not shown) and mature E1 glycoprotein but appeared to contain PE2′ in place of the E2 glycoprotein present in virions from CHO-K1 cells (Fig. 6, lanes 1 and 2). The amount of ^{35}S label present in each virus preparation, indicative of the number of total virus particles produced by each cell, was similar. The kinetics of [^{3}H]uridine incorporation into SV virions confirmed that the release of virus particles from both wild-type CHO-K1 and mutant RPE.40 cells occurred at similar rates (Fig. 7). These data demonstrated that RPE.40 cells produce and release noninfectious virus particles which contain PE2′ and also that SV virions can be formed and released from the cell without cleavage of PE2 to E2 and E3.

We investigated whether trypsin might be able to cleave the PE2′ in the noninfectious virions from RPE.40 cells and render them infectious. Trypsin treatment had little or no effect on virions from CHO-K1 cells, but it modified the abnormal virions from RPE.40 cells so that they now con-

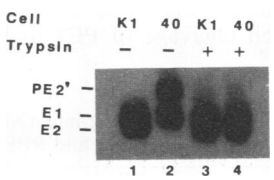

FIG. 6. SDS-PAGE analysis of purified SV virions from CHO-K1 and RPE.40 cells. Supernatant from ^{35}S-labeled SV-infected cells was collected 12 h postinfection. Virions were purified by sucrose gradient centrifugation and analyzed by SDS-PAGE without further treatment (lanes 1 and 2) or following a 30-min digestion with trypsin (10 μg/ml) at 37°C (lanes 3 and 4). Counts in virions in gradient peaks were 1,553 and 1,522 cpm/10^{6} cells for CHO-K1 and RPE.40 cells, respectively.

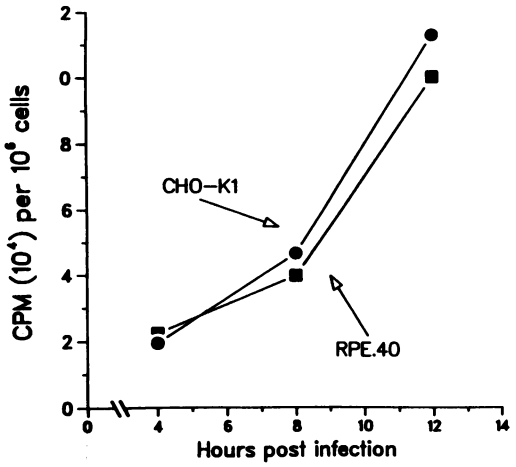

FIG. 7. Incorporation of [^{3}H]uridine into released SV. CHO-K1 and RPE.40 cells were infected with SV at 10 to 20 PFU/ml. At 2 h postinfection, the medium was replaced with MEM containing dactinomycin (4 μg/ml) and [^{3}H]uridine (5 μCi/ml). At the indicated times, culture supernatant was removed, and virions were precipitated by the addition of an equal volume of 10% trichloroacetic acid. Precipitates were collected on Whatman GFA glass fiber filters and washed, and radioactivity was determined by liquid scintillation spectrometry.

tained mature E2 glycoprotein rather than PE2′ (Fig. 6, lanes 3 and 4). Trypsin treatment of unlabeled SV preparations from RPE.40 cells caused a 20-fold increase in the number of infectious SV particles (Table 1). Similar activation of noninfectious virions was also obtained by treating SFV and CV released from RPE.40 cells with trypsin.

Presley and Brown (23) and Russell et al. (27) recently reported two conditions under which mature SV virions containing precursor glycoprotein PE2 were released from BHK (Syrian hamster) cells. These virions differed from those produced in RPE.40 cells in that they were fully infectious for BHK cells. We ascertained that this was not due to a difference in the cell lines by comparing plaque titers of SV produced in CHO-K1 and RPE.40 cells on BHK-21 cells. SV produced by the CHO-K1 cells readily infected BHK-21 cells to titers equal to or greater than those produced in CHO-K1 cells. However, only 5 to 8% as many infectious virus were produced by RPE.40 cells as were produced by CHO-K1 cells.

To further verify that the presence of uncleaved E2 precursor protein was directly responsible for the noninfectivity of strain AR339 produced in RPE.40 cells, we examined the infectivity of the strains of SV used by Russell et al. (27) in their studies, S.A.AR86 and S12, a mutant of S.A.AR86 in which cleavage of PE2 to E2 is blocked by

TABLE 1. Effect of trypsin on the infectivity of alphavirus preparations from CHO-K1 and RPE.40 cells

Cell	Trypsin added	Virus production (10^6 PFU/ml)		
		SV	SFV	CV
CHO-K1	−	40	60	240
	+	44	70	260
RPE.40	−	1.5	5.6	4.2
	+	29	36	70

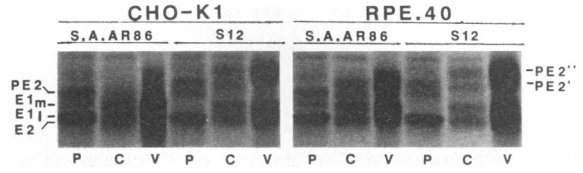

FIG. 8. Analysis of glycoproteins of SV strains S.A.AR86 and S12 produced in CHO-K1 and RPE.40 cells. Cells were infected with 10 to 20 PFU/cell and incubated at 37°C in F-12 growth medium containing dactinomycin (4 μg/ml). At 8 h postinfection, cells for extract preparation were pulsed as described in the legend to Fig. 1, and radioactivity was chased for 10 min. Cells for virus preparation were cultured for an additional 12 h in the above medium containing 25 μCi of Trans^{35}S-label per ml. P, Pulse; C, chase; V, released viruses.

mutation. We found that, unlike the case with AR339, RPE.40 cells had no resistance to either strain S.A.AR86 or its mutant. Endpoint titrations and plaque assays showed equal infectivity on CHO-K1 and RPE.40 cells. Infected cells were pulse-chase labeled with [^{35}S]methionine, as was done with strain AR339, and viral proteins were analyzed by SDS-PAGE and autoradiography (Fig. 8). CHO-K1 cells processed S.A.AR86 glycoproteins normally, producing mature E1 and E2 proteins both in cell extracts and in released virions. Infected RPE.40 cells again accumulated a protein that migrated more slowly than PE2, a PE2′, that was found in cell extracts and in released virions. CHO-K1 and RPE.40 cells were identical in their processing of the glycoproteins of mutant strain S12. A protein that migrated even more slowly than PE2′ (labeled PE2″ in Fig. 8) was apparent in cell extracts and released virions. We assume that this protein migrates more slowly because the mutation in S12 created an additional glycosylation site in PE2 (27). Inherent differences in the SV strains thus account for the differences we have observed in the infectivity of virions bearing uncleaved PE2 glycoprotein.

DISCUSSION

In this article we report that RPE.40, a mutant strain of CHO-K1 cells selected for resistance to *Pseudomonas* exotoxin A and cross-resistant to alphaviruses (21), fails to cleave the SV structural precursor glycoprotein PE2 to produce mature glycoprotein E2. RPE.40 cells generate instead a modified PE2, which we have designated PE2′, which has a slightly reduced electrophoretic mobility in SDS gels and which is incorporated into released virus.

A number of other cell strains that are resistant to SV and concomitantly resistant to various toxic proteins have been reported, but RPE.40 is unique in its phenotype. RPE.40 cells have a very pronounced resistance to SV and CV (100- to 10,000-fold more resistant than CHO-K1 cells) a lesser resistance to SFV (10-fold), and no resistance to vesicular stomatitis virus (21). Unlike the virus- and toxin-resistant CHO strains reported by Robbins et al. (26) and Presley et al. (24), they are not deficient in acidification of endosomal or lysosomal compartments. When tested by an acridine orange uptake method (4), vesicles isolated from RPE.40 postnuclear supernatant acidified as well as those from CHO-K1 (20a). RPE.40 cells have no resistance to diphtheria toxin, as do the SV-resistant CHO mutants of Mento and Siminovitch (20), and no resistance to ricin, as do the L cell mutants of Gottlieb et al. (8).

RPE.40 cells release a reduced number of infectious

virions, although normal yields of viral RNA are produced, indicating that they are not defective in mechanisms involved in virus entry or uncoating. In support of this, we have observed that SV preparations produce the same number of plaques on RPE.40 cells as on wild-type CHO-K1 cells; however, the plaque size is greatly reduced on RPE.40 cells (data not shown), indicating that RPE.40 produces some infectious virus or that virus at the cell surface is activated to a limited extent.

We demonstrated that the immature SV glycoproteins PE2 and $E1_i$ were produced in similar amounts in both the wild-type CHO-K1 and mutant RPE.40 cells. In addition, immature, high-mannose E1 was processed to its mature form, containing complex sugars and sialic acid, by both of these cell strains. This processing resulted in an apparent decrease in the electrophoretic mobility of the E1 glycoprotein. These results are in agreement with the findings of Bonatti and Cancèdda (2), who concluded that maturation of the E1 glycoprotein is accompanied by a decrease in the electrophoretic mobility of this protein. These authors identified the different forms of the E1 glycoprotein with molecular weight suffixes. Since the apparent molecular weights may not be identical for all strains of SV, in this study we refer to these forms by using the designations mature and immature and the abbreviations $E1_m$ and $E1_i$. In pulse-chase experiments, we observed that the conversion of PE2 to E2 in wild-type cells and of PE2 to PE2′ in RPE.40 cells occurred at similar rates. In addition, radiolabeled PE2 was chased at the same rate in both cell strains, suggesting that RPE.40 cells were not defective in their ability to transport PE2 out of the endoplasmic reticulum. We did not observe any abnormal accumulation of PE2 in the mutant RPE.40 cell.

Under the conditions of SDS-PAGE used in these experiments, mature E1 and E2 glycoproteins did not separate completely. Also, in pulse-chase experiments, the appearance of radiolabeled E2 was partially obscured by pulse-labeled immature E1, which had virtually the same electrophoretic mobility. Each viral glycoprotein was positively resolved, however, by the use of SV E1 and E2 antibodies. Since Western blotting detected the total amount of each viral glycoprotein present in infected cells, we were able to draw the following conclusions. (i) Equal numbers of RPE.40 cells and CHO-K1 cells infected with SV contained similar amounts of immature E1, mature E1, and PE2. (ii) The amount of PE2′ in RPE.40 cells was comparable to the amount of mature E2 present in CHO-K1 cells. (iii) The amount of PE2′ or E2 greatly exceeded the amount of PE2 in these cells. (iv) RPE.40 cells contained no detectable mature E2. These results indicated that the defect in production of infectious SV by RPE.40 cells was due to a failure to process PE2 to E2.

Detailed analysis showed that production of PE2′ requires passage of the PE2 glycoprotein into, if not through, the Golgi complex. It was determined that RPE.40 cells are able to glycosylate SV glycoproteins normally and that PE2′ is a form of PE2 in which the oligosaccharides have been correctly processed to the complex form but the proteolytic cleavage to produce E2 and E3 has not occurred. Despite this, RPE.40 and CHO-K1 cells produced and released virions at similar rates, although virions from RPE.40 cells were composed of C-protein, mature E1, and PE2′ with no measurable E2.

Knipfer and Brown (16) have recently shown that PE2 can be cleaved to E2 while still in an endo H-sensitive (high mannose) state and therefore is not wholly dependent upon the processing of the glycoprotein side chains. They suggested the possibilities that PE2 might encounter host cell enzymes capable of converting it to E2 while still in the rough endoplasmic reticulum and that this event might actually be virus mediated rather than cell mediated. Our work with RPE.40 leads us to think that the cleavage is not virus mediated, or we would expect to get normal infectious virus production in this cell. We have not been able to detect any abnormal condition, such as impaired acidification of vesicles, that might affect a virus-mediated proteolysis.

Since we detected no E2 in RPE.40, we can assume that PE2 has not encountered a functional cleavage enzyme in either the endoplasmic reticulum or the Golgi stacks. PE2′ does occur normally in CHO-K1 cells. It forms transiently in SV-infected CHO-K1 cells when pulse-chase experiments are performed at 8 h postinfection, but is not detected in virus-infected cell extracts labeled at 5 h postinfection. It is possible that early in the virus replicative cycle, PE2′ is formed but then is very rapidly cleaved to E2 and E3. Later in the replicative cycle, due to cellular changes which have occurred, PE2′ is not cleaved as efficiently and therefore can be observed in infected-cell extracts. These cellular changes may be due to the lack of host cell protein synthesis, to the amount of PE2 being produced, or to other changes induced by the virus.

It has been known for some time that the F glycoprotein of Sendai and Newcastle disease viruses is produced as a precursor glycoprotein, F_0, and that proteolytic cleavage of F_0 to F is not required for formation and release of virions from infected cells (22, 30). Similiarly, cleavage of the hemagglutinin glycoprotein of influenza A virus is necessary for infectivity but is not a precondition for virion assembly or expression of hemagglutinating activity (15). In each case, the viruses released into the medium can be converted to an infectious form by treatment with trypsin. Therefore, we investigated the ability of noninfectious SV virions produced by RPE.40 cells to be activated by trypsin treatment. Trypsin treatment increased the number of plaque-forming units in a preparation of SV released from RPE.40 cells by approximately 20-fold. In addition, the virions treated with trypsin contained mature E2 glycoprotein, while the amount of PE2′ was greatly reduced. Trypsin treatment had little or no effect on the infectivity of SV produced in CHO-K1 cells. Trypsin activation of noninfectious SFV and CV virions from RPE.40 cells confirmed that the defect in production of infectious alphaviruses in RPE.40 cells is the direct result of failure to cleave the precursor to the E2 glycoprotein.

Until recently it was generally believed that cleavage of PE2 to E2 was a required step in the formation and release of SV (31). Presley and Brown (23) and Russell et al. (27) showed that under certain conditions, infectious SV virions that contain PE2 can be released from BHK cells. Presley and Brown (23) studied virions produced when cells were exposed to monensin. These virions were not entirely devoid of E2 glycoprotein. The ratio of PE2 to E2 was 75:25, possibly providing enough E2 to insure infectivity. Russell et al. (27) studied mutants of SV strain S.A.AR86 which were mutated at the cleavage site in PE2, blocking production of E2 and causing incorporation of PE2 into virions. S.A.AR86 differs from AR339, the strain of SV that we used, in 147 point mutations, causing 22 amino acid differences. It is also virulent for adult mice, whereas most other SV strains, including AR339, are not. We tested the infectivity in CHO-K1 and RPE.40 cells of S.A.AR86 and its mutant S12, in which asparagine has been substituted for serine in position 1 of E2, creating a new N-linked glycosylation site

and blocking cleavage of PE2. We found these strains to be fully infectious in either cell whether their virions contained normal E2 or uncleaved PE2 glycoprotein. Virus strain differences, therefore, decide whether SV requires normal E2 in order to be infectious. Our observations also indicated that the virions of mutant strain S12 probably contain a derivative of PE2 in which the oligosaccharides have been completely processed to their complex form rather than PE2, as was reported (27).

We cannot be certain whether the AR339 virions produced by RPE.40 cells are of low (5 to 8% of normal) virulence or avirulent unless PE2′ is cleaved, as this cleavage might be accomplished, for a fraction of the virions, by a cell surface enzyme or by lysosomal enzymes from deteriorating cells. We do know that trypsin cleavage of their PE2′ greatly increases their infectivity.

The most likely explanation for the failure of RPE.40 cells to cleave PE2 is that they have a defect in the enzyme activity responsible for this cleavage. For several alphaviruses, including SV, the amino acid sequence at the site of cleavage between the E2 and E3 glycoproteins has been determined (5, 7, 14, 29). For each virus examined, cleavage occurs following two consecutive basic amino acids, either lysine-arginine or arginine-arginine. An additional arginine residue is separated from the cleavage site by a single amino acid which may be basic or uncharged. This Arg-X-Arg/Lys-Arg sequence has also been determined to be the cleavage site for several other viral glycoproteins, including the HA glycoprotein of myxoviruses, the F glycoprotein of paramyxoviruses, the pr95 glycoprotein of retroviruses, and the prM glycoprotein of flaviviruses (34). We are investigating the ability of RPE.40 cells to cleave a variety of these precursor viral glycoproteins to their mature forms.

How might a defect in an intracellular protease activity explain the resistance of RPE.40 cells to *Pseudomonas* toxin? There is good evidence that the genetic lesion responsible for lack of cleavage of PE2 is also responsible for the high resistance of RPE.40 cells to *Pseudomonas* toxin. RPE.40 was selected from recently cloned, mutagenized CHO-K1 cells by a single exposure to *Pseudomonas* toxin. This type of toxin-resistant mutant occurred with a frequency of approximately 10^{-5} in cells mutagenized with ethylmethane sulfonate. We have isolated six such strains and, by complementation analysis, shown them to have identical genetic lesions. All are concomitantly resistant to SV. In addition, a cell strain with the same phenotype as RPE.40 and shown to have the same genetic lesion by complementation analysis has been isolated from L929 mouse cells (20a). We are considering two possibilities to explain the toxin resistance. The protease may be required to process a protein necessary for *Pseudomonas* toxin binding or uptake, or it may be involved in intracellular activation of the toxin. Studies are in progress to address these questions.

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