Events in the Endoplasmic Reticulum Abrogate the Temperature Sensitivity of Sindbis Virus Mutant *ts23*

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Temperature-sensitive mutations in proteins produced at or heated to a nonpermissive temperature render the proteins defective in some aspect of their maturation into functional entities. The characterization of temperature-sensitive mutations in model proteins, such as virus membrane proteins, has allowed the elucidation of critical events in the maturation of virus membranes as well as in the intracellular folding, processing, and transport of membrane proteins in general. We have used a transport-defective, temperature-sensitive mutant of Sindbis virus, *ts23*, which has two amino acid changes in the envelope protein E1, to further examine requirements placed upon the glycoproteins for their export to the plasma membrane. Pulse-chase experiments in which we utilized the transport inhibitors monensin and brefeldin A allowed us to synthesize and assemble the glycoproteins of *ts23* into export-competent heterodimers at the permissive temperature while concurrently blocking their export to the cell surface. After removal of the inhibitors and a shift to the nonpermissive temperature, we assayed for protein transport, cell-cell fusion, and infectious-particle production. Taken together, the data show that the irreversible loss of the temperature-sensitive phenotype of *ts23* can be correlated with the folding of E1 and the formation of export-competent PE2-E1 heterodimers in the endoplasmic reticulum. Furthermore, we have found that E1 pairs with PE2 to form the heterodimer prior to the completion of E1 folding.

The maturation of virus membrane glycoproteins is a multistep process resulting in the production of oligomeric complexes which are capable of binding to, and initiating fusion with, a cell membrane. For Sindbis virus (the prototype of the alphaviruses) the product of this complex process is a metastable heterotrimeric spike composed of three E2-E1 heterodimers (34, 38). Eighty heterotrimers are organized into an icosahedral lattice within the host-derived lipid bilayer (1, 8, 38). Formation of the envelope protein lattice occurs during envelopment at the cell surface, where repeated interactions between the T=4 nucleocapsid and the cytoplasmic domain of E2 organize trimers of E2-E1 heterodimers into a T=4 icosahedral lattice that is stabilized by lateral associations among the trimers (1, 8, 9, 38, 39). The heterotrimeric spike and the strict organization of the spikes into an icosahedral lattice are characteristics which distinguish alphaviruses from many other membrane-containing viruses.

Correct oligomerization and processing of alphavirus membrane proteins are essential to achieve the very specific protein-protein associations required for envelope formation and virus maturation. During the development of the heterotrimer, E1 transiently interacts with the endoplasmic reticulum (ER) luminal chaperone protein BiP and folds into a compact, highly constrained configuration through a number of unstable disulfide-bridged intermediates (34, 35). As E1 folds within the ER, it interacts with PE2 (the precursor to E2) to form a heterodimer with a half-life of approximately 4 min at 37°C (35). PE2-E1 heterodimers are exported from the ER to the Golgi apparatus, where their N-linked oligosaccharides are processed to more-complex forms (44). Within the trans-Golgi network (TGN), PE2 is processed to mature E2 when the signal sequence of PE2 is cleaved by a furin-like proteinase (2,

10). This proteolytic event is therefore a reliable assay for the export of PE2/E2-E1 heterodimers to the TGN.

The role of PE2-E1 heterodimer formation in the export of Sindbis virus glycoproteins is not clear. Experiments in which the E1 glycoprotein has been expressed in the absence of PE2 have produced conflicting results. One study produced data suggesting that E1 expressed in the absence of PE2 in CV1 cells exited the ER and reached the cell surface with kinetics similar to that of E1 synthesized in the presence of PE2 (33). In contrast, another study concluded that E1 synthesized in the absence of PE2 in BHK cells was not expressed at the cell surface, although export of E1 from the ER was not assayed directly (16). Experiments with the alphavirus Semliki Forest virus have suggested that the ability to export E1 from the ER is dependent upon P62 synthesis and heterodimer formation (19, 32).

Sindbis virus mutant *ts23* is a complementation group D mutant whose E1 sequence contains two amino acid substitutions, an alanine-to-threonine change at position 106 and an arginine-to-glutamine change at position 267 (46). At the nonpermissive temperature (39.5°C) the alterations in E1 prevent the export of both E1 and PE2 from the ER (15, 22, 42). At a high temperature E1 of *ts23* is misfolded and incorporated into large, disulfide-stabilized aggregates containing E1, PE2 (6), and, as we have shown previously, the molecular chaperone BiP (35). At the permissive temperature (28°C) *ts23* grows to a high titer (~10⁹ PFU/ml), and the mature virus is not inactivated by subsequent incubation at 39.5°C (5, 48). The thermostability of mutant E1 within the envelope of mature *ts23* virions at 39.5°C (5, 48) suggests that the temperature-sensitive phenotype associated with E1 is lost at the time of or before nucleocapsid envelopment.

To further characterize the events that are responsible for the loss of E1 temperature sensitivity in *ts23* and to identify critical steps in alphavirus membrane protein maturation, we have performed temperature shift experiments with *ts23*-in-

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TABLE 1. Cell-cell fusion of *ts23*-infected BHK cells at 28 and 39.5°C

Expt	Description ^a	Fusion score ^b
1 (control)	7-h infection at 28°C; 30-min chase at 28°C	++++
2 (control)	7-h infection at 28°C; 30-min chase at 39.5°C	++++
3 (monensin control)	Monensin added 2 h postinfection at 28°C; fusion 7 h postinfection	—
4	Following protocol for expt 3, monensin removed 7 h postinfection; 2-h chase at 28°C	+++
5	Following protocol for expt 3, monensin removed 7 h postinfection; 2-h chase at 39.5°C	++++
6 (BFA control)	BFA added 2 h postinfection at 28°C; fusion 7 h postinfection	—
7	Following protocol for expt 6, BFA removed 7 h postinfection; chase at 28°C	++++
8	Following protocol for expt 6, BFA removed 7 h postinfection; 2-h chase at 39.5°C	+++
9 (monensin-BFA control)	Monensin added 2 h postinfection at 28°C; at 7 h postinfection, 15-min chase in the presence of monensin and BFA at 28°C; monensin removed; 90-min chase in the presence of BFA at 28°C	—
10	Following protocol for expt 9, 90-min chase in BFA; BFA removed; 2-h chase at 28°C	++++
11	Following protocol for expt 9, 90-min chase in BFA; BFA removed; 2-h chase at 39.5°C	++++

^a BHK cells were infected with *ts23* at a multiplicity of infection of 100 PFU per cell. Where indicated, BFA was used at 5 µg/ml and monensin was used at 25 µM. Where indicated, BFA and monensin were removed by washes with PBS. All chases were in the presence of 50 µg of cycloheximide per ml.

^b Fusion was scored as described in Materials and Methods. All monolayers were assayed for fusion competence at the temperature of their last incubation.

fecting BHK cells. It is shown below that loss of the temperature-sensitive phenotype of *ts23* coincides, temporally, with the folding of E1 into more-compact disulfide-bridged conformations and with the formation of the PE2-E1 heterodimer. The results support the model that heterodimer formation is required for E1 export from the ER in Sindbis virus-infected cells (16) and suggest that PE2 interacts with E1 of *ts23* to produce a thermostable heterodimer. The data also demonstrate that the formation and/or stabilization of the heterodimer does not occur immediately after translocation of PE2 and E1 into the ER but is correlated with the folding of E1 into more-compact isoforms.

MATERIALS AND METHODS

Materials. [³⁵S]methionine-cysteine was purchased from DuPont New England Nuclear (Boston, Mass.). Polyclonal E1-E2-specific (R50) and E1-specific (R47) antisera were produced in rabbits against gel-purified envelope proteins. Antibodies were isolated from rabbit antiserum with a 1-ml HiTrap protein A column (Pharmacia). Brefeldin A (BFA) was purchased from Epicenter Technologies (Madison, Wis.) and stored as a stock solution of 1 mg/ml in methanol at -20°C. BFA was added to the cell culture media at a concentration of 5 µg/ml. Monensin was purchased from Calbiochem (La Jolla, Calif.), and a 1 mM stock ethanol solution was stored at -20°C. Monensin was added to the cell culture media at a concentration of 25 µM. Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Cell culture and virus. BHK-21 cells were grown as described previously (22). *ts23* was propagated in BHK-21 cells as previously described (41). Original virus stocks of *ts23* were obtained from the laboratory of Elmer Pfefferkorn (Dartmouth Medical College). *ts23* virus titration was determined by plaque assay at 28°C as previously described (41).

Infection with *ts23*, radioactive labeling, and temperature shifts. Subconfluent monolayers were pretreated with actinomycin D as previously described (22) and were infected with 50 to 100 PFU of *ts23* per cell for 60 min at room temperature. Minimum Eagle's essential medium containing 10% fetal calf serum, 5% tryptose phosphate broth, and 2 mM glutamine was then added, and the cells were incubated at 28° for 4.25 h. The infected cells were starved for 15 min in methionine- and cysteine-free minimum Eagle's essential medium containing 1% fetal calf serum, 5% tryptose phosphate broth, and 2 mM glutamine prior to the pulse. At the end of the starvation period cells were metabolically labeled with [³⁵S]methionine-cysteine (50 µCi/ml) as previously described (40). Chase medium consisted of minimum Eagle's essential medium containing 50 µg of cycloheximide per ml and 10 mM (10×) cold methionine. Radiolabeled proteins were chased and/or temperature shifted by washing the monolayers with 28 or 39.5°C chase medium and by incubation at the specified temperatures.

Immunoprecipitation. Monolayers were lysed in cold TNT lysis buffer (pH 7.2) containing 1% Triton X-100 (Sigma), 10 mM Tris, 150 mM NaCl, and 0.2 mM phenylmethylsulfonyl fluoride. The nuclei were pelleted and discarded, and the supernatants were treated with 200 µl of antiserum conjugated to cyanogen bromide-activated Sepharose 4B beads in lysis buffer. Samples were agitated overnight at 4°C. The bead-antibody complexes were washed three times in cold lysis buffer prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Monolayers lysed in preparation for immunoprecipitation and analysis by nonreducing SDS-12.5% PAGE were washed in cold phosphate-buffered saline (PBS) (4°C) and 20 mM *N*-ethylmaleimide (NEM) prior to lysis in TNT buffer containing 20 mM NEM.

PAGE. Prior to analysis by reducing SDS-PAGE, each sample was mixed 1:1 with 2× reducing sample buffer and heated to 100°C for 5 min (43). Equal counts or equal volumes were loaded onto denaturing SDS-10.8% polyacrylamide gels as previously described (22). Prior to analysis by nonreducing SDS-12.5% PAGE, each sample was mixed 1:1 with 2× nonreducing sample buffer and heated to 80°C for 5 min (34). Fluorography was performed as described elsewhere (3), and dried gels were exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.). The autoradiograph of the nonreducing gel in Fig. 5b was digitized with a Sharp JX-325 high-resolution scanner and enhanced by using Adobe Photoshop 2.5 software to increase contrast between bands, and all bands shown in Fig. 5b were enhanced equally. Densitometry of the autoradiograph in Fig. 5b was performed on the original nonenhanced gel.

Fusion of *ts23*-infected BHK cells. Fusion competence was determined as previously described (31). Low-pH-mediated fusion from within was scored as follows: —, no fusion; +, fewer than 10 nuclei per polykaryon and less than 25% of cells fused; ++, more than 10 nuclei per polykaryon and less than 50% cells fused; +++, more than 10 nuclei per polykaryon and more than 50% cells fused; and +++++, more than 95% cells fused (see Table 1).

Immunofluorescence microscopy of *ts23*-infected BHK cells. Cells were grown in detachable microscope slide flaskettes. At the designated time the cells were fixed in 3% formaldehyde at 4°C for 6 h and then permeabilized for 10 min with 1% Triton X-100 in PBS. Cells were then washed in PBS two times for 5 min each time and blocked for 1 h in 10% Carnation powdered milk in PBS (PBS milk). Slides were then incubated for 2 h in polyclonal E1-E2 antiserum. The cells were washed three times for 5 min each time with PBS-milk and then incubated for 1 h in fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. Cells were then washed three times for 10 min each time in PBS, and coverslips were placed on the slides with mounting medium (5% glycerol in water). The slides were viewed with an Ultima laser scanning confocal microscope (Meridian Instruments, Inc., Okemos, Mich.). Multiple optical sections (64 by 64 µm each, at 320 by 320 pixels and 0.2 µm per pixel) were scanned at 0.5-µm increments in the *z* axis with a Z-stepper motor. These sections were then combined into maximum-intensity projections to permit visualization of the fluorescence in the entire scanned volume by using the Ultima system software (Meridian Instruments, Inc.).

RESULTS

Thermostability of exported heterodimers after return to the ER. The ability of *ts23* mutant glycoprotein E1 to remain in a functional, correctly folded conformation at 39.5°C in the protein lattice of mature virus indicates that at some point between the translocation of the envelope proteins into the ER and the maturation of virus at the plasma membrane, the temperature-sensitive phenotype of E1 is lost. Glycoprotein E1 is matured in the ER by passing through a series of unstable disulfide-bridged intermediates (34, 35). The development of the stable form of E1 occurs temporally with the formation of the PE2-E1 heterodimer, suggesting that PE2 stabilizes E1 in the heterodimer (34). These data suggested that the folding of

FIG. 1. Immunofluorescence of *ts23*-infected BHK cells treated with monensin and BFA. BHK cells seeded onto microscope slide flaskettes were infected with *ts23* and incubated at 28°C. At 2 h postinfection the cells were treated with 25 μ M monensin. (a) At 6 h postinfection one monolayer was chased in a mixture of cycloheximide, monensin (25 μ M), and BFA (5 μ g/ml) for 15 min. These cells were then washed with PBS at 4°C and chased for an additional 90 min at 28°C in a mixture of BFA and cycloheximide prior to cell fixation and permeabilization. (b) The other monolayer was chased in the presence of cycloheximide and monensin for 90 min prior to cell fixation and permeabilization. Polyclonal anti-E1-E2 antiserum was used as the primary antibody, and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G was used as the secondary antibody. Each panel is a maximum-intensity projection of a series of sections viewed with a confocal laser scanning microscope as described in Materials and Methods.

E1 and its association with PE2 abrogate E1 temperature sensitivity in mutant *ts23*.

In the following experiments we utilized the well-characterized intracellular transport inhibitors monensin (18, 20, 21, 40) and BFA (7, 12, 24, 25) to determine if PE2-E1 heterodimers formed and exported to the Golgi at permissive temperature in the presence of monensin would exhibit the temperature-sensitive transport phenotype if first returned to the ER in the presence of BFA prior to incubation at the nonpermissive temperature. The protocol employed was similar to that used by de Silva et al. (11) in their study of G protein in cells infected with vesicular stomatitis virus *ts045*. Monensin, a carboxylic ionophore, was used to retain and accumulate virus glycoproteins in the medial Golgi compartments (20, 21). BFA was used to induce the retrograde transport of the *cis*, medial, and *trans* stacks of the Golgi apparatus with their associated proteins to the ER (7, 12, 24, 25). Accumulation of envelope proteins in the medial Golgi apparatus and the ER following treatment with monensin and BFA, respectively, was confirmed by immunofluorescence confocal microscopy of monensin- or monensin-BFA-treated *ts23*-infected BHK cells. Monensin-BFA-treated cells exhibit the diffuse reticular and perinuclear staining of the ER previously shown to be characteristic of cells treated with BFA alone (12, 24, 25) (Fig. 1a). Monensin-treated cells exhibit the enhanced, localized staining previously shown to be indicative of the Golgi apparatus in alphavirus-infected cells treated with monensin (21) (Fig. 1b). These data confirm that in the presence of monensin protein transport is arrested in the Golgi apparatus. When BFA is added to these cells, the proteins are returned to the ER.

Cells infected with *ts23* were pulse-labeled for 5 min at 28°C with [³⁵S]methionine-cysteine and chased at 28°C in the presence of cycloheximide and 25 μ M monensin (to trap exported proteins in the medial Golgi apparatus) for 90 min (Fig. 2a). BFA was then added at 28°C, and monensin was removed. After a 90-min chase in BFA, the temperature was shifted to 39.5°C and the BFA was removed. The chase was continued for specified times, at which point the monolayers were lysed and the envelope proteins were immunoprecipitated and analyzed by reducing SDS-PAGE (Fig. 2b).

Processing of PE2 to E2 occurs at the TGN (10), and a chase

in 25 μ M monensin completely blocks the export of PE2 beyond the medial Golgi apparatus and as a result prevents the processing of PE2 to E2 (Fig. 2b, lane 1) (20, 40). Since a characteristic of the phenotype of *ts23* at the nonpermissive temperature is the retention of both PE2 and E1 within the ER (15, 22), the appearance of E2 indicates export of the labeled envelope proteins to the TGN after release from the effects of monensin and BFA (Fig. 2b). The processing of PE2 to E2 occurs within 45 min, at 39.5°C, after removal of monensin and BFA (Fig. 2b, lanes 2 to 4). The decrease in the amount of labeled envelope protein recovered in the gel following the extended chases (90 and 150 min) after BFA removal probably results from the maturation of *ts23* glycoproteins at 39.5°C into infectious virus (see Table 1 and Fig. 4). Mature virus purified from the media collected at the end of the 150-min chase contained labeled E1 and E2 (data not shown). These data demonstrate that the envelope proteins that were exported to the Golgi apparatus during the chase in monensin remain export competent even if they are returned to the ER after addition of BFA and are incubated at the nonpermissive temperature. The temperature-sensitive mutants of Sindbis virus show some limited mutant phenotype even at the permissive temperature, and the protein recovered in Fig. 2b, lane 4, probably represents a portion of the pulse-labeled PE2 and E1 protein which remained in the ER in a temperature-sensitive state during the chase in both monensin and BFA.

E1 becomes thermostable prior to arrival in the *cis* Golgi apparatus. The folding of E1 and the formation of the PE2-E1 heterodimer occur in the ER in the presence of BFA (34). BFA, if added 15 min prior to a pulse-label, will effectively prevent the export of the labeled proteins from the intermediate compartment of the Golgi apparatus, resulting in the retention and accumulation of the labeled proteins within the ER (12, 25). To determine if the abrogation of E1 temperature sensitivity occurs in the ER, we assayed the processing of PE2 to E2 (a TGN event) in *ts23* envelope proteins which were pulse-labeled and chased in the presence of BFA at 28°C in order to allow for their retention within the ER prior to the removal of BFA and continuation of the chase at 39.5°C. BHK cells infected with *ts23* were treated with BFA for 15 min prior to and during a 5-min label with [³⁵S]methionine-cysteine at

FIG. 2. (a) Flow chart for the experiment shown in panel b. PT, permissive temperature; NPT, nonpermissive temperature. (b) *ts23* envelope proteins exported to and retained in the Golgi apparatus at the permissive temperature prior to their return to the ER remain export competent and do not misfold in the ER upon a shift to the nonpermissive temperature. The experiment was conducted as diagramed in panel a. Immunoprecipitations were done using polyclonal E1-E2 antiserum. Equal volumes from each sample were analyzed by reducing SDS-10.8% PAGE. The appearance of E2 confirms export of PE2-E1 heterodimers through the TGN. Numbers at the bottom indicate durations (in minutes) of chases. Mon, monensin.

28°C. The cells were then chased in cycloheximide for 2.5 h at 28°C in the presence of BFA. At the end of a 2.5-h chase, the BFA was removed by washing the monolayers three times in PBS warmed to 39.5°C. The chase was then continued for the specified times at 39.5°C (Fig. 3). At the end of each chase time the monolayer was lysed and immunoprecipitated with E1-E2-specific antiserum for analysis by SDS-PAGE. As in the previous experiment, the appearance of E2 was used to assay for E1 export from the ER. E2 is not present immediately after the chase in BFA, confirming that the envelope proteins were not exported (Fig. 3, lane 1). However, after the removal of BFA and a continuation of the chase at 39.5°C, conversion of PE2 to E2 occurs (Fig. 3, lanes 2 to 4). We have previously shown that Sindbis virus envelope proteins synthesized and chased in the presence of BFA fold less efficiently (35). Therefore, as stated above, the PE2 and E1 which remain after a 90-min chase are probably representative of a population of the pulse-labeled protein that was unable to fold into a thermostable conforma-

tion and was retained in the ER in a temperature-sensitive state (Fig. 3, lane 4). This conclusion is supported by our observations that the PE2 and E1 which remain after the 90-min chase at 39.5°C (Fig. 3, lane 4) exist as a disulfide stabilized aggregate when analyzed by SDS-PAGE under non-reducing conditions (data not shown) and that the effect of BFA on the cleavage of PE2 is completely reversible for wild-type virus-infected cells subjected to the same conditions (data not shown). These data suggest that the event(s) which allows the export competence of E1 at the nonpermissive temperature occurs in the ER prior to the export of E1 to the Golgi apparatus.

Cell surface expression of fusion-competent E1. E1 contains the activity required for fusion of the viral membrane with the target cell membrane (23, 37, 47). Cell-cell fusion from within (FFWI) can be induced in Sindbis virus-infected cells and is dependent upon the expression of functional envelope proteins at the cell surface (31). Thus, the ability to induce FFWI in Sindbis virus-infected cells can be used as an assay for the presence of functional E1 at the cell surface. BHK cells infected with *ts23* at 28°C fuse efficiently at 7 h postinfection (31; this study). Sindbis virus *ts23* envelope proteins synthesized and maintained at 39.5°C do not induce FFWI because they are retained in the ER (31).

The data presented above suggested that once PE2-E1 heterodimers are exported from the ER, they remain in a thermostable conformation at 39.5°C. In the following cell-cell fusion experiments the delivery (chase in the presence of cycloheximide) of functional (fusion-competent) *ts23* E1-E2 heterodimers to the cell surface at either 28 or 39.5°C was determined for (i) envelope proteins synthesized and exported at 28°C and then subjected to a 30-min chase (28 or 39.5°C) in cycloheximide (controls) (Table 1, experiments 1 and 2) (ii) envelope proteins that were synthesized and retained by BFA in the ER at 28°C prior to the removal of BFA and a chase (28 or 39.5°C) in cycloheximide (Table 1, experiments 6, 7, and 8), (iii) envelope proteins that were synthesized and retained in the medial Golgi apparatus at 28°C by monensin treatment prior to the removal of monensin and a chase (28 or 39.5°C) in cycloheximide (Table 1, experiments 3, 4, and 5) and (iv) envelope proteins that were synthesized and retained in the medial Golgi apparatus in the presence of monensin and subsequently returned to the ER at 28°C via a chase in the presence of BFA and cycloheximide prior to the removal of BFA and a chase (28 or 39.5°C) in cycloheximide (Table 1, experiments 9,

FIG. 3. The abrogation of *ts23* temperature sensitivity occurs in the ER in the presence of Golgi components. BHK cells infected with *ts23* were pulse-chased at 28°C in the presence of 5 µg of BFA per ml as described in the text. After the chase at 28°C, the BFA was removed and the chase was continued for 45, 60, and 90 min at 39.5°C. At the end of each chase the monolayers were lysed and immunoprecipitated with E1-E2 antiserum. Equal counts were analyzed by reducing SDS-10.8% PAGE. The appearance of E2 confirms the export of PE2-E1 heterodimers through the TGN.

10, and 11). Cell surface expression and the functionality of E2-E1 heterodimers were determined by assaying for FFWI at 28 or 39.5°C (Table 1).

As expected, FFWI does not occur immediately after treatment with either monensin or BFA or both inhibitors, since each of these drugs prevents the expression of E1 at the cell surface (Table 1, experiments 3, 6, and 9). FFWI does occur at either 28 or 39.5°C after a 7-h infection at 28°C (Table 1, experiments 1 and 2). FFWI also occurs in cells chased in cycloheximide at 28 or 39.5°C after treatment with monensin or BFA or sequential treatments of monensin and BFA at 28°C if the agents are removed (Table 1, experiments 4, 5, 7, 8, 10, and 11). These data confirm, as suggested in the results presented above, that significant amounts of both E1 and E2, if allowed to fold within the ER at the permissive temperature, are able to overcome the export defect which is a characteristic of *ts23* at the nonpermissive temperature. These results also demonstrate that the fusogenic activity of the export-competent heterodimer is not lost at the nonpermissive temperature.

Infectious Sindbis virus production after monensin and/or BFA treatment. The function of Sindbis virus proteins in virus penetration differs in several ways from their function in low-pH-mediated FFWI (4), raising the possibility that the proteins present at the cell surface (described in the preceding section) do not form virions capable of infecting cells. Therefore, we determined if *ts23* envelope proteins that were retained in the ER, retained in the Golgi apparatus, or returned from the Golgi apparatus to the ER could be incorporated into infectious virus if chased to the cell surface at 39.5°C. BHK monolayers were infected with *ts23* for 4.5 h at 28°C, at which time the monolayers were washed three times in PBS prior to chases in the presence of cycloheximide at 28°C. One set of monolayers was chased for 3 h with media containing BFA and cycloheximide, in order to retain a population of envelope proteins within the ER. The other set was chased in media containing monensin and cycloheximide for 1.5 h and then chased in BFA and cycloheximide for an additional 1.5 h in order to increase the proportion of previously exported heterodimers returned to, and retained within, the ER. After the chase in the presence of transport inhibitors, the monolayers were washed three times with PBS, and the final wash was collected. The monolayers were then chased in the presence of cycloheximide for an additional 4 h at 28°C (positive control) or 39.5°C in the absence of either transport inhibitor. At the termination of the 4-h chase, the media from the monolayers and the final wash (to remove inhibitor) were assayed for infectious virus by plaque assay. The amount of infectious virus present in the wash or produced during the 39.5°C chase was converted to a percentage of the amount of virus produced at 28°C (positive control) (Fig. 4).

The monolayers in which virus was matured at 39.5°C after the chase in BFA produced 47.5% of the virus produced in the positive control (Fig. 4). These data indicate that infectious virus is produced at 39.5°C from envelope proteins retained in the ER by a chase in BFA at 28°C prior to a chase in the absence of BFA at 39.5°C and support the contention that the loss of E1 temperature sensitivity occurs prior to export of the proteins from the ER. The monolayers in which virus was matured at 39.5°C after the sequential chases in monensin and BFA at 28°C produced 66% of the infectious virus produced by the positive control (Fig. 4). The amount of infectious virus in the final wash from all samples prior to chase (4%) confirms that virus was produced during the 4-h chase after removal of the transport inhibitors. It is significant that the amount of virus relative to the control produced at 39.5°C after a chase in both monensin and BFA is larger than that produced after a

FIG. 4. Infectious *ts23* virus production after a 3-h chase in BFA or sequential 1.5-h chases in monensin (Mon) and BFA. BHK monolayers were infected with *ts23* (multiplicity of infection of 100) at 28°C for 4.5 h prior to a 3-h chase at 28°C in the presence of cycloheximide and the transport inhibitors monensin and BFA as described in the text. The inhibitors were removed by washing the monolayers three times in PBS warmed to either 28 or 39.5°C. The third wash to remove the inhibitors was analyzed for virus production by plaque assay (Wash). The chase in cycloheximide was continued at 28°C (positive controls) or 39.5°C for 4 h, at which time the media were removed and virus production was determined by plaque assay. Virus titers from the monolayers shifted to 39.5°C were converted to a percentage of the virus titers from the monolayers incubated at 28°C for the entire chase period.

chase in BFA alone. This result suggests that more E1 attains the ability to be exported at 39.5°C when chased in monensin than when chased in BFA at 28°C and that the envelope proteins which were able to fold correctly and exit the ER during the chase in monensin were incorporated into infectious virus even after their return to, and retention in, the ER prior to incubation at the nonpermissive temperature.

Events associated with development of E1 thermostability. The folding of E1 into its most compact conformation, which has been designated E1_f, and the formation of the PE2-E1 heterodimer occur in the ER prior to export to the Golgi apparatus (34, 35, 42, 49). We have recently demonstrated that E1 progresses through a series of folding steps prior to the formation of the heterodimer (34, 35). When the glycoproteins of *ts23* are synthesized at the nonpermissive temperature, the defect in E1 prevents the export of both E1 and PE2 from the ER (15, 22). It is therefore possible that the folding of E1 within the ER and/or the formation of the PE2-E1 heterodimer at permissive temperature is the event(s) abrogating the temperature sensitivity of E1 in *ts23*. The following experiments were designed to determine if heterodimer formation and/or the formation of specific E1 folding intermediates correlates in time with the abrogation of E1 temperature sensitivity.

The rate of formation of export-competent, thermostable E1 was determined by pulse-labeling BHK cells infected with *ts23* for 5 min at 28°C. The monolayers were then chased in the presence of cycloheximide for a total of 60 min, and in five of six monolayers the temperature was shifted to 39.5°C at specified times after the pulse (Fig. 5a). At the end of each chase, monolayers were lysed and immunoprecipitated envelope proteins were analyzed by reducing SDS-PAGE. The appearance of E2 (produced by proteolytic processing in the TGN) in any one of the shifts indicates that at the time of the shift to 39.5°C a population of thermostable, export-competent E1 had already been established. E2 was first detected if the shift to

39.5°C occurred 10 min after the 5-min pulse (Fig. 5a). This confirms that it takes no longer than 15 min at 28°C for export-competent E1 to form.

In order to investigate if heterodimer formation and/or a specific E1 folding step coincided with the abrogation of E1 temperature sensitivity, BHK monolayers infected with *ts23* were pulse-labeled for 5 min at 28°C and chased in the presence of cycloheximide at 28°C for specified times. At the end of each chase, monolayers were washed for 10 min in cold PBS (4°C) containing 20 mM NEM (to block free thiols and inhibit artifactual disulfide formation or rearrangements) and lysed in the presence of 20 mM NEM. Lysates were immunoprecipitated with E1-specific polyclonal antiserum, and the precipitated protein was analyzed by nonreducing SDS-PAGE to resolve E1 folding intermediates (34) (Fig. 5b). Coimmunoprecipitation of PE2 with E1 was used to assay for heterodimer formation (29, 35).

An increase in the coprecipitation of PE2 with E1 is detected at 10 min after the 5-min pulse at the permissive temperature (Fig. 5b and c). The increase in PE2 coprecipitation coincides in time both with the formation of more-compact, disulfide-bridged E1 folding intermediates E1 β and E1 γ and with the abrogation of E1 temperature sensitivity (Fig. 5a and b). Under reducing conditions E1 migrates as a single species (data not shown). It is significant that in each lane (Fig. 5b) the amount of PE2 which coprecipitates with E1 is approximately equivalent to the amount of E1 β and E1 γ present (Fig. 5c). Since E1 β is the precursor to E1 γ (34), we suspect that heterodimer formation is likely occurring concurrent with or after folding of E1 into its E1 β conformation. These data support the hypothesis that the folding of E1 within the ER into the more compact β and γ conformations and/or the formation of the PE2-E1 heterodimer is the event(s) required for the abrogation of E1 temperature sensitivity.

FIG. 5. (a) Processing of *ts23* PE2 to E2 synthesized at 28°C and shifted to 39.5°C during the chase. BHK monolayers infected with *ts23* were pulse-labeled for 5 min at 28°C as described in Materials and Methods. The monolayers were chased in cycloheximide for a total of 60 min, and the incubation temperature was shifted from 28 to 39.5°C at the specified times. At the end of the chase, monolayers were lysed and immunoprecipitated with polyclonal E1-E2 antiserum. Equal counts of immunoprecipitates were analyzed by reducing SDS-10.8% PAGE. The incubation times during the chase are given below the gel. (b) E1 β and E1 γ formation and PE2 coimmunoprecipitation with E1 at the permissive temperature correspond to envelope protein acquisition of export competence. BHK monolayers infected with *ts23* were pulse-labeled at 28°C as described in Materials and Methods and chased at 28°C in cycloheximide for the indicated times. Chases were terminated by lysis of the monolayers in the presence of 20 mM NEM, which was followed by immunoprecipitation with anti-E1 polyclonal antiserum. Equal volumes of immunoprecipitates were analyzed by nonreducing SDS-12.5% PAGE. The autoradiograph was scanned as described in Materials and Methods. All protein bands were enhanced equally. (c) Quantitation of PE2 coprecipitating with E1 isoforms β and γ . For each lane in panel b the integrated optical densities of PE2 and the individual E1 isoforms resolved in the gel (nonenhanced) were determined and converted to a percentage of the total amount of immunoprecipitated protein. PE2 bands were normalized for their larger total methionine-cysteine content. The amount of PE2 per lane is compared with the amount of E1 β and E1 γ per lane. For each lane in panel b the amount of PE2 which coprecipitates with E1 is roughly equivalent to the amount of E1 β and E1 γ isolated.

DISCUSSION

The folding, processing, and oligomerization of proteins within the ER occur both during and after translocation of the newly synthesized polypeptide(s) (13, 17). For proteins that move through the secretory pathway, events occurring in the ER play a critical role in the formation of export-competent structures. Recently, Sindbis virus E1 glycoprotein folding within the ER prior to or during heterodimer formation has been shown to involve the progression of E1 through a series of unstable intermediates which differ in the number and/or arrangement of their disulfide bonds (34). The formation of correctly folded PE2-E1 heterodimers within the ER may be necessary for the export of Sindbis E1 glycoproteins from the ER and is a prerequisite for the formation of the specific protein-protein associations that will result in the T=4 lattice of the Sindbis virus envelope as virus assembly takes place (1, 8, 38, 42, 45, 49).

Several functions have previously been ascribed to the PE2-E2 glycoprotein. The E2 endodomain is required for envelopment of the nucleocapsid (26, 27), the E2 ectodomain is involved in attachment of virus to the host cell (14, 30, 36), and the cleavage of PE2 to E2 plays a role in the activation of the metastable spike (14, 28). Our data support the contention that heterodimer formation is required for the export of E1 from the ER (16). The data indicate that the formation and/or

stabilization of the heterodimer correlates with the folding of E1 into two of its more compact isoforms, E1 β and E1 γ . This is significant because we have previously reported that only the most compact of the E1 species, E1 ϵ , is stable with respect to its disulfide bonds and that the conversion of E1 ϵ into the metastable form found in mature virus corresponds in time with the cleavage of PE2 into E2 (34). These observations taken together with the correlation between heterodimer formation and the loss of *ts*23 E1-induced temperature sensitivity suggest that PE2 stabilizes E1. It is also possible that upon heterodimer formation PE2 provides a scaffold on which E1 is able to complete its folding from E1 β into the stable E1 ϵ conformation.

Our results also indicate that after the folding of E1 into the more compact isoforms E1 β and E1 γ and the formation of the heterodimer in the ER at 28°C, incubation of *ts*23-infected cells at the nonpermissive temperature does not cause the misfolding or retention of the heterodimer by the quality control mechanisms of the ER. The data further demonstrate that the ability of heterodimers to exit the ER, to reach the cell surface, to induce cell-cell fusion, and to be incorporated into infectious virus is not disrupted by incubations at the nonpermissive temperature once the heterodimers have been formed.

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