The Formation of Intramolecular Disulfide Bridges Is Required for Induction of the Sindbis Virus Mutant ts23 Phenotype

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The Sindbis virus envelope protein spike is a hetero-oligomeric complex composed of a trimer of glycoprotein E1-E2 heterodimers. Spike assembly is a multistep process which occurs in the endoplasmic reticulum (ER) and is required for the export of E1 from the ER. PE2 (precursor to E2), however, can transit through the secretory pathway and be expressed at the cell surface in the absence of E1. Although oligomer formation does not appear to be required for the export of PE2, there is evidence that defects in E1 folding can affect PE2 transit from the ER. Temperature-sensitive mutant ts23 of Sindbis virus contains two amino acid substitutions in E1, while PE2 and capsid protein have the wild-type sequence; however, at the nonpermissive temperature, both E1 and PE2 are retained within the ER and can be isolated in protein aggregates with the molecular chaperone GRP78-BiP. We previously demonstrated that the temperature sensitivity for ts23 was lost as oligomer formation took place at the permissive temperature, suggesting that temperature sensitivity is initiated early in the process of viral spike assembly (M. Carleton and D. T. Brown, J. Virol. 70:952–959, 1996).

Experiments described herein investigated the defects in envelope protein maturation that occur in ts23-infected cells and which result in retention of both envelope proteins in the ER. The data demonstrate that in ts23-infected cells incubated at the nonpermissive temperature, E1 folding is disrupted early after synthesis, resulting in the rapid incorporation of both E1 and PE2 into disulfide-stabilized aggregates. Furthermore, the aberrant E1 conformation which is responsible for induction of the ts phenotype requires the formation of intramolecular disulfide bridges formed prior to E1 association with PE2 and the completion of E1 folding.

Sindbis virus, the prototype of the Alphavirus genus, has a complex three-dimensional structure containing two nested and interconnected icosahedral protein shells, both of which have T = 4 icosahedral symmetry (1, 12, 13, 37, 38). The inner shell is comprised of 240 copies of the capsid protein (C) and the single, positive strand of infectious viral RNA (12, 13, 37, 38). The outer protein shell contains 240 copies each of two envelope integral membrane glycoproteins, E1 and E2, assembled into 80 envelope protein spikes composed of a trimer of E1-E2 heterodimers (1, 12, 38). The organization of the viral spikes into the outer icosahedral protein shell is driven by interactions between the C protein in the assembled nucleocapsid and the carboxyl-terminal domain of E2 during the process of envelopment (27–29, 38). The stability of the envelope protein lattice is maintained by lateral associations between the viral spikes which are in turn stabilized by intramolecular disulfide bridges within glycoprotein E1 (1, 2, 9, 18, 36, 38).

Viral spike assembly is a multistep process that originates with the translation of the three structural proteins from a subgenomic RNA into a single polyprotein in the order NH2-C-PE2 (E3-E2)-6K-E1-COOH (reviewed in references 42 and 44). Capsid protein autoproteolytically cleaves itself from the developing polypeptide, exposing a hydrophilic signal sequence at the NH2-terminal end of PE2, allowing the targeting and translocation of the polypeptide into the membranes of the rough endoplasmic reticulum (ER) (5). The junctions between 6K and PE2 and E1 are cut by the ER resident signal peptidase (26), and E1 folds through a series of folding intermediates designated E1α, E1β, and E1γ, which are unstable with respect to their intramolecular disulfide bridges, and then into its stable and most compact E1ε conformation (32). Prior to PE2-E1 association and spike formation, E1 folding from E1α to E1β is characterized by the transient association of E1 with GRP78-BiP, an increase in resistance to proteolytic degradation by trypsin, and the formation of an epitope recognized by the conformation-specific monoclonal antibody SIN33 (10, 33). Assembly of PE2-E1 heterodimers (spike promoters) occurs concurrent with or immediately prior to the assembly of the spike complex (capsomere) (34). Spike assembly occurs after E1β formation and is required for E1 acquisition of resistance to the membrane-permeative reducing agent dithiothreitol (DTT) (8–10, 34) and export of E1 from the ER (8, 10, 23, 31). Prior to the arrival of the spike at the cell surface, PE2 is processed to E2 by a furin-like proproteinase resident within the trans-Golgi network (3, 14, 46). Therefore, the cleavage of E3 from PE2 can be used to assay the export of the spike from the ER in ts23-infected cells (8).

Sindbis virus mutant ts23 is a complementation group D mutant (7) whose E1 sequence contains two amino acid substitutions, an alanine-to-threonine substitution at position 106 and an arginine-to-glutamine substitution at position 267 (43). When the envelope proteins of ts23 are synthesized at the nonpermissive temperature (39.5°C), the sequence alterations in E1 result in the retention of both E1 and PE2 in the ER, as indicated by the failure to process PE2 to E2 and the continued sensitivity of both envelope glycoproteins to endoglycosidase H (25). The retention of ts23 envelope proteins in the ER at the nonpermissive temperature is accompanied by the association of both E1 and PE2 with BiP (33) which contains the ER retention sequence KDEL (35). ts23 virus produced at the permissive temperature (28°C) is not inactivated by subsequent incubation at 39.5°C (7, 47). The loss of the ts phenotype

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during virus maturation at the permissive temperature corresponded temporally with the folding of E1 into the E1β conformation and PE2-E1 oligomer formation (8). We have expanded upon these previous studies by investigating the specific events involved in the induction of ts23 temperature sensitivity in order to characterize the defect(s) in ts23 protein maturation which results in the association of E1 and PE2 with BiP and their retention within the ER at the nonpermissive temperature. Evidence is presented which indicates that at the nonpermissive temperature, both E1 and PE2 are incorporated into disulfide-stabilized aggregates. The data also demonstrate that induction of the ts phenotype is dependent upon intramolecular disulfide bond formation occurring during the initial folding of E1. Furthermore, the inability of E1 at the nonpermissive temperature to acquire a conformation which is resistant to trypsin proteolysis and recognized by the conformation-specific monoclonal antibody SIN33 indicates that the two mutations in the E1 protein of ts23 disrupt an early step in E1 folding occurring prior to the productive association of E1 with PE2 and the formation of the viral spike.

MATERIALS AND METHODS

Materials. [35S]Methionine-cysteine was purchased from DuPont New England Nuclear (Boston, Mass.). Polyclonal E1-E2-specific and E1-specific antisera were produced in rabbits against gel-purified envelope proteins. Antibodies were isolated from rabbit antiserum with a 1-m1 HiTrap protein A column (Pharmacia). Monoclonal anti-E1 antibody (SIN33) was kindly provided by A. L. Schmaljohn (U.S. Army Medical Research Institute of Infectious Diseases). N-Ethylmaleimide (NEM) was purchased from Sigma Chemical Co. (St. Louis, Mo.) and used at a concentration of 20 mM. A 0.2 M (100 mM) stock NEM–buffered saline (PBS) solution was made immediately prior to use. DTT was purchased from Sigma Chemical Co. and used at a concentration of 5 mM. A 0.5 M (500 mM) stock DTT–ethanol solution was made immediately prior to use. Trypsin was purchased from Worthington Biochemical Corp. (Freehold, N.J.) and used at a concentration of 10 μg/ml. A 1-mg/ml stock trypsin-PBS solution was made immediately prior to use. Soybean trypsin inhibitor was purchased from Worthington Biochemical Corp. and used at a concentration of 30 μg/ml.

Cell culture and virus. BHK-21 cells were cultured at 37°C in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (GIBCO), 5% nonessential amino acids, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Original virus stocks of heat-resistant Sindbis virus and ts23 were originally provided by E. R. Pfeifferkorn (Dartmouth Medical College) and were propagated in BHK-21 cells as previously described (40).

Infection with virus and radioactive labeling. Subconfluent monolayers were pretreated with actinomycin D as previously described (39) and were infected with either the ts-deficient or the heat-resistant Sindbis virus or ts23 per 60% confluence at 37°C. After 1 h of adsorption at the appropriate temperature, the monolayers were fed with minimum Eagle's medium containing 1% fetal bovine serum, 5% trypsin–phosphate broth, and 2 mM L-glutamine (GIBCO). The original virus stocks of heat-resistant Sindbis virus and ts23 were provided by C. Schmaljohn (U.S. Army Medical Research Institute of Infectious Diseases). The neuraminidase activity was neutralized with 5 mM N-acetyl-D-glucosamine (GIBCO). The cells were allowed to sit for 5 min at 4°C prior to immunoprecipitation.

Preparation of cell lysates for centrifugation and immunoprecipitation. After completion of the chase, cells were washed with ice-cold PBS. Cells were then solubilized with 750 μl of 10% triton X-100 (Sigma) 10 min. TMS 150 mM NaCl, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The nuclei were pelleted and discarded. A solution of disulfide cross-links and/or disulfide bridge reformation was prevented by including 20 mM NEM in both the PBS wash and the lysis buffer. Cell lysates were subjected to density gradient centrifugation as described below, or envelope proteins were immunoprecipitated from the cell lysates at 4°C using one of the following three antisera, polyclonal anti-E1, polyclonal anti-E2 or anti-E1 monoclonal antibody SIN33, as previously described (8, 10, 33). Polyclonal sera were conjugated to cyanogen bro-}

Density gradient centrifugation. Cell lysate (750 μl) was layered onto a five-step (700 μl each) 5 to 25% sucrose gradient (wt/wt) with a 60% sucrose cushion. Sucrose solutions were made in TNE (30 mM Tris, 100 mM NaCl, 1.25 mM EDTA; pH 7.2) containing 1% Triton X-100 and 0.2 mM PMSF. The 5-ml gradients were centrifuged at 40,000 rpm (Sorvall ORD 255) for 24 h at 4°C and were then fractioned from the bottom into 15 fractions (350 μl). One hundred microliters of each fraction was mixed with 100 μl of reducing (in the presence of 2-mercaptoethanol) or nonreducing (in the absence of 2-mercaptoethanol) 2× SDS gel sample buffer in preparation for SDS-PAGE. Lysates from Sindbis virus-infected, pulse-labeled BHK cells and purified [35S]methionine-cysteine-labeled mature virus were subjected to density gradient centrifugation as described above. Immunoprecipitation of PE2 or E2 with the E1-specific monoclonal antibody SIN33 from gradient fractions was used to differentiate between heterodimer- and monomer-containing fractions (11).

Trypsin proteolysis. Trypsin sensitivity assays were performed on proteins from lysates prepared in the absence of PMSF. Lysates were incubated at 37°C for 10 min either in the presence or absence of 10 μg of trypsin per ml. Soybean trypsin inhibitor was then added to the lysates at a concentration of 30 μg/ml and allowed to sit for 5 min at 4°C prior to immunoprecipitation.

Gel electrophoresis. Prior to analysis by SDS-PAGE, each sample was mixed 1:1 with either 2× nonreducing or reducing sample buffer and heated at 80°C for 4 min (nonreduced samples) or at 100°C for 5 min (reduced samples). Equal counts or equal volumes (as noted in figure legends) from each sample were loaded onto polyacrylamide gels (PAGs), and electrophoresis was carried out as previously described (8, 32, 39). Fluorography was performed as previously described (6), and dried gels were exposed to Kodak BMR film (Eastman Kodak Co., Rochester, N.Y.). Identification of the different E1 folding intermediates was determined by analyzing the mobility of each E1 intermediate on an SDS-PAG compared to the mobilities of standard molecular weight markers on the same SDS-PAG.

RESULTS

Ts23 envelope proteins synthesized at the nonpermissive temperature form disulfide cross-linked aggregates. The aggregation and retention of misfolded viral envelope proteins within the ER has been described for several viral proteins (reviewed in reference 17). The exposure of hydrophobic domains on the surfaces of incompletely or incorrectly folded proteins is believed to induce protein aggregation by allowing the formation of premature intermolecular interactions (reviewed in references 17 and 21). Protein aggregates may become covalently cross-linked if free sulfhydryl groups are also exposed (reviewed in references 17 and 21). In addition, BiP has been shown to bind sequences of hydrophobic amino acids; therefore, BiP recognition and binding to protein aggregates could be mediated by the exposure of hydrophobic sequences which would normally be hidden within a protein's native structure (4, 20). In one such instance, the G protein of vesicular stomatitis virus mutant ts045 when synthesized at the nonpermissive temperature is unable to form homotrimers, does not acquire a complete set of disulfide bonds, and does not present specific conformational epitopes which are present in the native structure (15, 16). The consequence of this deficient folding is the incorporation of G protein and BiP into large noncovalently associated aggregates which are retained in the ER (30).

Transient BiP-E1 associations occur prior to the assembly of the spike in Sindbis virus-infected cells (33). BiP may also interact with PE2, but such interactions have not been detected during the course of a normal infection (33). However, BiP has been shown to enter into a stable association with PE2 during a ts23 infection in which the envelope proteins were pulse-labeled and chased at the nonpermissive temperature (33). The formation of ts23 envelope protein aggregates at the nonpermissive temperature was first reported by Rice and Strauss (41) and could provide a rationale for the production of wild-type PE2 within the ER and the association of BiP with both E1 and PE2 (33). Further analysis of the protein aggregates formed at the nonpermissive temperature in ts23-infected cells was conducted in the following experiments. ts23-infected or Sindbis virus-infected BHK cells were pulse-labeled

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with [35S]methionine-cysteine at 39.5°C for 5 min followed by a chase at 39.5°C in the presence of cycloheximide for 45 min. At the end of the chase, the infected monolayers were lysed, and sedimentation analysis of infected-cell lysates on sucrose gradients was performed. The gradients were fractionated from the bottom, and 100 µl of each fraction was analyzed by nonreducing (NR) and reducing (R) SDS-PAGE. Nonreduced gels were exposed to film 1.5 times longer than reducing gels to enhance the signal for aggregates (Agg) resident at the top of the gel.

FIG. 1. E1 and PE2 are isolated as disulfide-stabilized aggregates from cell lysates of ts23-infected BHK cells incubated at the nonpermissive temperature. BHK cells infected with either ts23 (A and B) or Sindbis virus (SVHR) (C and D) were pulse-labeled and then chased in the presence of cycloheximide at the nonpermissive temperature as described in the text. At the end of the chase, infected-cell monolayers were solubilized with detergent. Each cell lysate was subjected to sucrose gradient sedimentation analysis as described in Materials and Methods. Gradients were fractionated from the bottom, and 100 µl of each fraction was analyzed by nonreducing (NR) and reducing (R) SDS-PAGE. Nonreduced gels were exposed to film 1.5 times longer than reducing gels to enhance the signal for aggregates (Agg) resident at the top of the gel.

It has been shown that the disulfide bridges resident in E1 folding intermediates undergo rearrangements if isolated in the absence of NEM (32). Therefore, it is possible that the disulfide cross-links within the aggregates recovered from the experiment above were not formed inside the cell but were due to aberrant disulfide bond formation occurring during or after cell lysis. This possibility was investigated in the following experiment. BHK cells infected with ts23 were pulse-labeled with [35S]methionine-cysteine at 39.5°C for 5 min and chased at 39.5°C in the presence of cycloheximide for the times shown in Fig. 2A. At the end of the chase, cell monolayers were lysed

lanes 1 to 7). The majority of the envelope protein resolved in the lighter fractions of the gradient containing lysate from ts23-infected cells pulsed and chased at the nonpermissive temperature is due to the incomplete migration of all protein aggregates into the heavier gradient fractions. This is supported by the fact that significant amounts of envelope protein are resolved from these fractions only when analyzed under reducing conditions. In addition, the gradient profile for ts23-infected BHK cells at the permissive temperature resembled that of Sindbis virus-infected cells, except that less PE2 had been processed to E2 during the 45-min chase due to the decrease in temperature (11). These data suggest that at the nonpermissive temperature, both E1 and PE2 are incorporated into large disulfide-stabilized aggregates in ts23-infected cells which are too large to be resolved under the nonreducing PAGE conditions depicted in Fig. 1A.
done under reducing conditions demonstrates that the protein aggregates contain both PE2 and E1. Furthermore, it is likely that domains are exposed within these aggregates which are recognized and bound by BiP. It is also of interest to note that small amounts of all the E1 folding intermediates are recovered in the presence of NEM with a small amount of fully folded E1 being isolated by 1 h of chase (Fig. 2A, lanes 4 to 6). These data indicate that in addition to the processing of a limited amount of PE2 to E2 at the nonpermissive temperature (Fig. 1B), a limited amount of E1 is also able to slowly fold correctly at the nonpermissive temperature. E1 has previously been shown to be the only E1 species which if isolated in the absence of NEM is stable with respect to its disulfide bonds (32). This observation is supported by the fact that in the absence of NEM, only E1 is present in the resolving gel analyzing the proteins of t23-infected cells under nonreduced conditions (Fig. 2A, lanes 2 and 3).

### Prevention of intramolecular disulfide bridge formation at the nonpermissive temperature blocks the induction of the temperature-sensitive phenotype.

It has previously been shown that the presence of 5 mM DTT during the synthesis and translocation of secretory proteins into the ER prevents the formation of nascent disulfide bonds (45). This observation holds true for t23 glycoproteins synthesized in the presence of 5 mM DTT at either 28 or 39.5°C in that they migrate with completely reduced envelope protein when analyzed by nonreducing SDS-PAGE (11). In addition, the thermoreversibility of the t23 phenotype is an inefficient process, as evidenced by the observations that t23 envelope proteins pulse-labeled at the nonpermissive temperature and then chased at the permissive temperature remain sensitive to endoglycosidase H digestion, sediment with smooth membranes of intermediate density, and exhibit little processing of PE2 to E2 (8, 19, 24, 25). These characteristics of t23 envelope proteins were utilized to determine if disulfide bridge formation is required for induction of temperature sensitivity. BHK cells infected with t23 were pulse-labeled with [35S]methionine-cysteine at 39.5°C for 5 min in the presence of DTT in order to prevent formation of disulfide bridges. At the end of the pulse, DTT was removed and a 28°C chase was initiated in the presence of cycloheximide and continued for the times indicated (Fig. 3). At the end of the chase, the monolayers were lysed, envelope proteins were immunoprecipitated with polyclonal anti-E1-E2 serum, and immunoprecipitated protein was analyzed by reducing SDS-PAGE (Fig. 3). The processing of PE2 to E2 was used as an indicator for the export of viral spikes from the ER and as a measure for the extent to which the mutant phenotype had been established (8). Immediately after the pulse, both E1 and PE2 were present in noncovalent aggregates which sedimented to the bottom of sucrose gradients in experiments similar to those described above (11). However, during the chase, these aggregates were resolved into monomers and heterodimers (11), and as shown in Fig. 3, the processing of PE2 to E2 begins to occur between 1 and 2.5 h of chase and is nearly complete by 5 h of chase. The increased time required for PE2 processing was also observed in control t23-infected cells in which the pulse in

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**FIG. 2.** E1 and PE2 of t23 are incorporated into disulfide-stabilized aggregates at the nonpermissive temperature. BHK cells infected with t23 were pulse-labeled at 39.5°C for 5 min and chased in the presence of cycloheximide for 0, 1, and 4 h. Infected-cell monolayers were lysed with detergent at the end of the pulse and the times indicated (Fig. 3). At the end of the chase, the monolayers were lysed, envelope proteins were immunoprecipitated with polyclonal anti-E1-E2 serum, and immunoprecipitated protein was analyzed by nonreducing SDS-PAGE (12.5% polyacrylamide) (A) or reducing SDS-PAGE (10.8% polyacrylamide) (B). The autoradiogram depicted in panel A was overexposed to enhance the signal of protein aggregates (Agg.) resident at the top of the gel.

Both in the absence or presence of the sulfhydryl-alkylating agent NEM in order to prevent aberrant disulfide bond formation and/or rearrangement (9, 32). Envelope proteins were immunoprecipitated with polyclonal anti-E1 serum, and the immunoprecipitated protein was analyzed by nonreducing (Fig. 2A) and reducing (Fig. 2B) SDS-PAGE. The absence immediately after the pulse of significant amounts of PE2 and E1 which can enter the resolving gel under nonreducing conditions (Fig. 2A, lanes 1 and 4) indicate that aggregate formation is rapid. Similar results in the presence of NEM (Fig. 2A, lanes 4 to 6) confirm that the formation of these aggregates is occurring within the cell and is not an artifact of disulfide rearrangements occurring during or after cell lysis. In addition, the ability to coprecipitate a significant amount of PE2 with polyclonal anti-E1 serum (Fig. 2B, lanes 1 to 6), which has previously been shown to disrupt normal PE2-E1 noncovalent associations (32), and to resolve these proteins by SDS-PAGE

### Methods.

1. Detection of E1 and PE2 in t23-infected cells.

   - Infected-cell monolayers were lysed with detergent at the end of the pulse (Fig. 1A).
   - Equal counts of immunoprecipitated protein were analyzed by nonreducing SDS-PAGE (Fig. 1B) and reducing (Fig. 2B) SDS-PAGE.
   - The absence of NEM is stable with respect to its disulfide bonds (32). This observation is supported by the fact that in the absence of NEM, only E1 is present in the resolving gel analyzing the proteins of t23-infected cells under nonreduced conditions (Fig. 2A, lanes 2 and 3).

   - Prevention of intramolecular disulfide bridge formation at the nonpermissive temperature blocks the induction of the temperature-sensitive phenotype. It is demonstrated above that production of t23 at the nonpermissive temperature results in the formation of disulfide bond-stabilized protein aggregates. We next attempted to determine if the formation of these aggregates were dependent upon the formation, at the nonpermissive temperature, of intramolecular disulfide bridges in E1 or if noncovalent associations between incorrectly folded E1 proteins, independent of intramolecular disulfide bridge formation, induce aggregate formation followed by disulfide bridge stabilization of the aggregate.

   - It has previously been shown that the presence of 5 mM DTT during the synthesis and translocation of secretory proteins into the ER prevents the formation of nascent disulfide bonds (45). This observation holds true for t23 glycoproteins synthesized in the presence of 5 mM DTT at either 28 or 39.5°C in that they migrate with completely reduced envelope protein when analyzed by nonreducing SDS-PAGE (11). In addition, the thermoreversibility of the t23 phenotype is an inefficient process, as evidenced by the observations that t23 envelope proteins pulse-labeled at the nonpermissive temperature and then chased at the permissive temperature remain sensitive to endoglycosidase H digestion, sediment with smooth membranes of intermediate density, and exhibit little processing of PE2 to E2 (8, 19, 24, 25). These characteristics of t23 envelope proteins were utilized to determine if disulfide bridge formation is required for induction of temperature sensitivity. BHK cells infected with t23 were pulse-labeled with [35S]methionine-cysteine at 39.5°C for 5 min in the presence of DTT in order to prevent formation of disulfide bridges. At the end of the pulse, DTT was removed and a 28°C chase was initiated in the presence of cycloheximide and continued for the times indicated (Fig. 3). At the end of the chase, the monolayers were lysed, envelope proteins were immunoprecipitated with polyclonal anti-E1-E2 serum, and immunoprecipitated protein was analyzed by reducing SDS-PAGE (Fig. 3). The processing of PE2 to E2 was used as an indicator for the export of viral spikes from the ER and as a measure for the extent to which the mutant phenotype had been established (8).

   - Immediately after the pulse, both E1 and PE2 were present in noncovalent aggregates which sedimented to the bottom of sucrose gradients in experiments similar to those described above (11). However, during the chase, these aggregates were resolved into monomers and heterodimers (11), and as shown in Fig. 3, the processing of PE2 to E2 begins to occur between 1 and 2.5 h of chase and is nearly complete by 5 h of chase. The increased time required for PE2 processing was also observed in control t23-infected cells in which the pulse in
are synthesized at 39.5°C in the presence of DTT. BHK cells infected with ts23 were pulse-labeled at 39.5°C for 5 min in the presence of DTT. At the end of the pulse, the chase was initiated and the DTT was removed by washing the monolayers at 28°C in the presence of cycloheximide. The chase was continued at 28°C for 0, 1, 2.5, and 5 h. At the end of the chase, the monolayers were lysed with detergent and envelope proteins were immunoprecipitated with polyclonal anti-E1-E2 serum. Equal counts of immunoprecipitated protein were analyzed by reducing SDS-PAGE (10.8% polyacrylamide). The processing of PE2 to E2 was assessed using E1-E2 serum and equal counts of immunoprecipitated protein were analyzed by nonreducing SDS-PAGE (10.8% polyacrylamide). The processing of PE2 to E2 was used to assay for export of virus spikes from the ER.

The observation that processing of glycoprotein PE2 to E2 for 0, 1, 2.5, and 5 h. At the end of the pulse, the chase was initiated and the DTT was removed by washing the monolayers at 28°C in the presence of cycloheximide. The chase was continued at 28°C for 0, 1, 2.5, and 5 h. At the end of the chase, the monolayers were lysed with detergent and envelope proteins were immunoprecipitated with polyclonal anti-E1-E2 serum. Equal counts of immunoprecipitated protein were analyzed by reducing SDS-PAGE (10.8% polyacrylamide). The processing of PE2 to E2 was used to assay for export of virus spikes from the ER.

The presence of DTT was carried out at the permissive temperature (11). The slowed processing of PE2 to E2 likely results directly from the exposure to DTT. It has been reported previously that the presence of DTT during the synthesis of envelope proteins P62 and E1 of the alphavirus Semliki Forest virus decreases their folding efficiency after DTT removal (45). The observation that processing of glycoprotein PE2 to E2 takes place if disulfide bridge formation at high temperatures is prevented suggests that the formation of intramolecular disulfide bonds within E1 is necessary for the induction of the ts23 phenotype.

**Formation of intramolecular disulfide bridges within E1 is necessary to establish** *ts* **23 temperature sensitivity.** We have previously shown that under wild-type conditions, a 5-min treatment with 5 mM DTT immediately after a pulse-label results in only partial reduction of E1 intramolecular disulfide bonds (9). However, if the chase is extended and the spike trimer is allowed to assemble after the pulse, E1 disulfide bridges become completely resistant to 5-min treatments with DTT (9). A similar experiment was conducted with ts23-infected BHK cells in order to determine the effect of short-term DTT treatment on both envelope protein aggregates and intramolecular disulfide bridges within E1 after a pulse-label and chase at the nonpermissive temperature (Fig. 4). BHK cells infected with ts23 were pulse-labeled with [35S]methionine-cysteine at 39.5°C for 5 min and chased for various times in the presence of cycloheximide at 39.5°C prior to a 5-min exposure to 5 mM DTT. The exposure to DTT was terminated by detergent solubilization of the infected-cell monolayer in the presence of NEM. Envelope proteins were immunoprecipitated from the cell lysates with polyclonal anti-E1-E2 serum, and immunoprecipitated protein was analyzed by nonreducing SDS-PAGE (Fig. 4).

DTT exposure at various times during the chase is able to reduce disulfide cross-links which stabilize the envelope protein aggregates formed at the nonpermissive temperature. This is indicated by the resolution of both PE2 and E1 in nonreduced SDS-PAGE after DTT exposure (Fig. 4). In addition, the presence of both reduced E1 and an E1 conformation which exhibits a mobility in SDS-PAGE similar to that of the earliest E1 folding intermediate E1α indicates that just as in a wild-type virus infection, a 5-min exposure to DTT immediately after the pulse does not completely reduce all E1 intramolecular disulfide bridges (Fig. 4) (9). However, extending the chase at the nonpermissive temperature prior to DTT exposure does not increase the resistance of E1 intramolecular disulfide bridges to DTT reduction, as is the case for a wild-type virus infection (Fig. 4) (9). Furthermore, if immediately after a 5-min pulse-label at the nonpermissive temperature, ts23-infected cells are treated with DTT, as in Fig. 4, lane 1, such that a portion of the labeled E1 is not completely reduced, removal of DTT and continuation of the chase at the permissive temperature do not significantly reverse the transport defect of the envelope proteins, as indicated by the inability to process PE2 to E2 (Fig. 5A). Complete processing of PE2 to E2 does occur if the pulse-label and DTT treatment are executed at the permissive temperature (Fig. 5B). These data indicate that in ts23-infected cells, a native spike trimer is not forming, the folding of E1 is aberrant, and the formation of E1 intramolecular disulfide bridges are necessary for the induction of the ts phenotype at the nonpermissive temperature.

Misfolded E1 does not acquire resistance to trypsin proteolysis or present the antigenic epitope recognized by E1 mono-
clonal antibody SIN33. The rapidity with which E1-PE2 are incorporated into disulfide bond-stabilized aggregates at the nonpermissive temperature suggests that the aberrant E1 conformation responsible for induction of the ts phenotype occurs early during E1 folding. The acquisition of E1 resistance to trypsin proteolysis and the development of an epitope recognized by the conformation-specific monoclonal E1 antibody, SIN33, are two events which occur as E1 folds from its Ea conformation into the E1b conformation prior to the formation of PE2-E1 oligomers (10). These two events can, therefore, be used to assay the extent of ts envelope protein E1 folding at the nonpermissive temperature.

BHK cells infected with ts23 were pulse-labeled with [35S]methionine-cysteine at 39.5°C for 5 min and chased at 39.5°C in the presence of cycloheximide for 15 min. The same protocol was followed for wild-type-virus-infected cells at 39.5°C. At the end of the chase, the infected monolayers were lysed with detergent and trypsin digestion was terminated by addition of an excess of soybean trypsin inhibitor to the lysates, and envelope proteins were immunoprecipitated with polyclonal anti-E1-E2 serum. Immunoprecipitates were analyzed by reducing SDS-PAGE (Fig. 6). Whereas E1 produced in wild-type virus infections at 39.5°C is resistant to trypsin proteolysis by 15 min after synthesis (Fig. 6), E1 produced in ts23-infected cells remains sensitive to trypsin throughout the chase period (Fig. 6). Furthermore, E1 synthesized at 39.5°C during a 5-min pulse-label of ts23-infected BHK cells and subsequently chased for 45 min at 39.5°C is not immunoprecipitated by the E1 monoclonal antibody SIN33 (Fig. 7, lane 1). In contrast, E1 recovered from ts23-infected cells incubated at the permissive temperature and E1 recovered from wild-type-virus-infected cells incubated at the nonpermissive temperature do display the epitope recognized by SIN33 and associate with PE2, as indicated by the coprecipitation of both PE2 and E1 with E1 monoclonal antibody SIN33 (Fig. 7, lane 1). The differences in the amounts of PE2 which are processed to E2 in ts23-infected cells at the permissive temperature and E1 recovered from wild-type-virus-infected cells incubated at the nonpermissive temperature do display the epitope recognized by SIN33 and associate with PE2, as indicated by the coprecipitation of both PE2 and E2 with E1 using SIN33 (Fig. 7, lanes 2 and 3). The differences in the amounts of PE2 which are processed to E2 in ts23-infected cells at the permissive temperature and in wild-type-virus-infected cells incubated at the nonpermissive temperature are due to the fact that the transport of the envelope glycoproteins to the trans-Golgi network is slower at 28°C (Fig. 7, lanes 2, 3, 5, and 6). These data further support the conclusion that the defect in envelope protein maturation in ts23-infected cells at the nonpermissive temperature occurs early during the folding of E1 prior to the formation of the E1β conformation but after the initial formation of an incomplete set of intramolecular disulfide bonds within E1.

FIG. 5. Incomplete reduction of E1 intramolecular disulfide bridges formed at the nonpermissive temperature in ts23-infected cells does not reverse induction of the temperature-sensitive phenotype. BHK cells infected with ts23 were pulse-labeled for 5 min at either the nonpermissive (A) or permissive (B) temperature followed by a chase in the presence of cycloheximide and DTT for 5 min at the temperature of the pulse. The chase was continued at the permissive temperature after removal of DTT for the times indicated. Infected-cell monolayers were lysed with detergent at the end of the chase. Envelope proteins were immunoprecipitated with polyclonal anti-E1-E2 serum, and equal counts of immunoprecipitated protein were analyzed by reducing SDS-PAGE (10.8% polyacrylamide). The processing of PE2 to E2 was used to assay for export of virus spikes from the ER.

FIG. 6. Glycoprotein E1 of ts23 does not acquire resistance to trypsin proteolysis at the nonpermissive temperature. BHK cells infected with ts23 or Sindbis virus (SVHR) were pulse-labeled for 5 min at the nonpermissive temperature and chased in the presence of cycloheximide for 15 min at the nonpermissive temperature. At the end of the chase, the cell monolayers were lysed with detergent. Lysates were incubated in the presence (+) or absence (−) of trypsin as described in Materials and Methods, and envelope proteins were immunoprecipitated with polyclonal anti-E1-E2 serum after trypsin exposure. Equal volumes of immunoprecipitated protein were analyzed by reducing SDS-PAGE (10.8% polyacrylamide).
DISCUSSION

The highly ordered structure of alphaviruses and the limited number of proteins involved in the formation of the two icosahedral shells of these enveloped viruses make Sindbis virus an excellent model for the study of enveloped virus assembly. The investigation of Sindbis virus envelope spike assembly has provided a better understanding of both virus- and cell-mediated mechanisms involved in the folding and transport of glycoproteins within the secretory pathway (9, 10, 32–34). This research was supported by grant AI14710 from the National Institutes of Health, by the Foundation for Research, Carson City, Nev., and by funds generally appropriated by the state of Texas to the Cell Research Institute.

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