JOURNAL OF VIROLOGY, Aug. 1996, p. 5541–5547 Vol. 70, No. 8
0022-538X/96/$04.00+0

Disulfide Bridge-Mediated Folding of Sindbis Virus Glycoproteins

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Received 7 March 1996/Accepted 9 May 1996

The Sindbis virus envelope is composed of 80 E1-E2 (envelope glycoprotein) heterotrimers organized into an icosahedral protein lattice with T=4 symmetry. The structural integrity of the envelope protein lattice is maintained by E1-E1 interactions which are stabilized by intramolecular disulfide bonds. Structural domains of the envelope proteins sustain the envelope's icosahedral lattice, while functional domains are responsible for virus attachment and membrane fusion. We have previously shown that within the mature Sindbis virus particle, the structural domains of the envelope proteins are significantly more resistant to the membrane-permeative, sulfhydryl-reducing agent dithiothreitol (DTT) than are the functional domains (R. P. Anthony, A. M. Paredes, and D. T. Brown, Virology 190:330–336, 1992). We have used DTT to probe the accessibility of intramolecular disulfides within PE2 (the precursor to E2) and E1, as these proteins fold and are assembled into the spike heterotrimer. We have determined through pulse-chase analysis that intramolecular disulfide bonds within PE2 are always sensitive to DTT when the glycoproteins are in the endoplasmic reticulum. The reduction of these disulfides results in the disruption of PE2-E1 associations. E1 acquires increased resistance to DTT as it folds through a series of disulfide intermediates (E1α, -β, and -γ) prior to assuming its native and most compact conformation (E1ε). The transition from a DTT-sensitive form into a form which exhibits increased resistance to DTT occurs after E1 has folded into its E1β conformation and correlates temporally with the dissociation of BiP-E1 complexes and the formation of PE2-E1 heterotrimers. We propose that the disulfide bonds within E1 which stabilize the protein domains required for maintaining the structural integrity of the envelope protein lattice form early within the folding pathway of E1 and become inaccessible to DTT once the heterotrimer has formed.

Sindbis virus, the prototype of the alphaviruses, is structurally different from many other enveloped viruses in that both the capsid protein (C) and the two envelope glycoproteins (E1 and E2) are assembled into icosahedral lattices with T=4 symmetry (1, 8, 9, 25, 26). The envelope of the mature Sindbis virus particle contains 240 copies of each glycoprotein organized into 80 heteroligomeric spike trimers of E1-E2 heterodimers (1, 8, 25). The heterotrimeric spikes are assembled within the endoplasmic reticulum (ER) and are subsequently exported through the secretory pathway (23). During transport PE2 (the precursor to E2) is processed to E2 within the trans-Golgi network by a furin-like proteinase, and the mature heterotrimers are expressed at the cell surface (3, 10, 38). It is the interaction of the carboxyl tails of E2 with the T=4 nucleocapsid during envelopement that drives the organization of the heterotrimeric spikes into the T=4 icosahedral lattice of the envelope (8, 9, 17, 18, 25). After assembly, E1-E1 interactions, stabilized by intramolecular disulfide bonds, play a critical role in maintaining the structural integrity of the envelope protein lattice (2).

During the process of spike assembly within the ER, E1 transiently associates with the molecular chaperone GRP78-BiP as it folds through a series of unstable disulfide intermediates (E1α, E1β, and E1γ) prior to assuming its most compact and stable conformation (E1ε) (21, 22). It is also in the ER that E1 associates with PE2 to form the heterodimer with a half-life ($t_{1/2}$) of approximately 4 min (22). Heterodimer formation occurs after E1 has folded into its E1β conformation (5, 23), precedes the completion of E1 folding (5, 23), and occurs concurrently with or immediately preceding heterotrimer formation (23). Heterodimer formation is also required for stabilizing E1 into an export-competent conformation (5, 12, 14, 19). Prior to the expression of the heterotrimer at the cell surface and its incorporation into the mature virus particle, E1ε becomes metastable with respect to its disulfide bonds (21). The conversion of stable E1ε into a metastable state as defined by the inability to isolate E1 as a single species correlates temporally with the processing of PE2 to E2 (21).

The membrane-permeative reducing agent dithiothreitol (DTT) is a well-established tool for the analysis of protein folding and conformation (33, 34). Anthony et al. (2) used DTT to probe the structural and functional domains of the Sindbis virus spikes in the mature virus particle. In that study it was determined that the function of the mature virus particle, the ability to infect the cell, was far more sensitive to inactivation by DTT than was the structural integrity of the envelope protein lattice. More recently DTT has been used to analyze viral envelope protein folding and oligomerization within the ER. It has been demonstrated that when the ER-resident G protein of vesicular stomatitis virus (VSV) and the hemagglutinin (HA) of influenza virus are subjected to short-term DTT exposure, the folding intermediates of these proteins are reduced and comigrate in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with completely reduced G protein or HA (33). The ability to reduce these intermediates is dependent upon metabolic energy and the intact nature of the ER (33). However, native, fully oxidized G protein and HA are resistant to reduction (33, 34). For VSV G protein, the acquisition of resistance to DTT correlates temporally with the assembly of native G protein within the ER

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into export-competent homotrimers (33). In the case of HA, acquisition of a DTT-resistant conformation immediately precedes and is independent of homotrimer formation (34).

To further characterize the folding of Sindbis virus glycoproteins we have utilized short-term DTT exposure to assay the relative accessibility of intramolecular disulfide bonds in PE2 and E1 as these proteins fold within the ER and are assembled into the heterotrimer. Our results suggest the E1 disulfides which stabilize structural domains in the mature virus particle and exhibit resistance to DTT form early during the folding of E1. The inaccessibility of these E1 disulfides to DTT appears to be mediated by the association of E1 with PE2 and the formation of the heterotrimer in the ER. These data also indicate that intramolecular disulfide bonds exist within PE2, that these disulfides remain sensitive to DTT after heterotrimer assembly, and that these disulfides may in part stabilize structural domains required for heterodimer formation.

MATERIALS AND METHODS

Materials. [^{35}S]methionine-cysteine was purchased from DuPont New England Nuclear (Boston, Mass.). Polyclonal E1 (R47)-specific antisera were produced in rabbits against gel-purified envelope protein. Polyclonal whole virus antiserum (R64) was produced in rabbits against purified whole virus. Antibodies were isolated from rabbit antiserum using a 1-ml HiTrap protein A column (Pharmacia). Monoclonal E1 antibody (SIN 33) 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 10× stock NEM–phosphate-buffered saline (PBS) solution was made immediately prior to use. DTT was purchased from Sigma and used at a concentration of 5 mM. A 100× stock DTT-ethanol solution was made immediately prior to use. 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 medium at a concentration of 25 μM.

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% tryptose phosphate broth, and 2 mM L-glutamine. Heat-resistant Sindbis virus was originally provided by E. R. Pfefferkorn (Dartmouth Medical College) and was propagated in BHK-21 cells as previously described (28).

Infection with heat-resistant Sindbis virus, radioactive labeling, and DTT treatment. Subconfluent monolayers were pretreated with actinomycin D as previously described (16) and were infected with 50 to 100 PFU of heat-resistant Sindbis virus per cell for 60 min at room temperature. Supplemented minimal essential medium was then added, and the cells were incubated at 37°C for 3.75 h. The infected cells were starved for 15 min in methionine- and cysteine-free minimal essential medium containing 1% fetal bovine serum, 5% tryptose phosphate broth, and 2 mM L-glutamine prior to the pulse. At the end of the starvation period cells were metabolically labeled with [^{35}S]methionine-cysteine (50 μCi/ml) as previously described (27). The chase medium consisted of minimal essential medium containing cycloheximide (50 μg/ml) and 10 mM (10×) concentration of cold methionine. Radiolabeled proteins were chased by washing the monolayers with chase medium after the pulse-label and incubation in the chase medium continued at 37°C for the specified times. Radiolabeled proteins were chased in the presence of DTT by dilution of the stock DTT-ethanol solution directly into the chase medium. Monensin-treated monolayers were exposed to monensin at the start of the starvation period, and monensin exposure was maintained throughout the pulse and chase.

Immunoprecipitation. At the end of the chase, monolayers were washed with and incubated for 10 min in ice-cold PBS containing 20 mM NEM and cycloheximide (50 μg/ml) at 4°C on ice. Each monolayer was lysed in 1 ml of ice-cold TNT lysis buffer (pH 7.2) containing 1% Triton X-100 (Sigma), 10 mM Tris, 150 mM NaCl, 20 mM NEM, and 0.2 mM phenylmethylsulfonyl fluoride. The nuclei were pelleted and discarded, and the supernatants were treated with either 50 μl of monoclonal E1 antiserum, 200 μl of polyclonal E1 antiserum, or 200 μl of whole virus antiserum. Polyclonal antiserum had been previously conjugated to cyanogen bromide-activated Sepharose 4B beads in lysis buffer. Samples were agitated for 2 h (E1 monoclonal) or overnight (polyclonal serum) at 4°C. E1 monoclonal immune complexes were recovered as previously described (22). The bead-antibody complexes were washed three times in cold lysis buffer prior to SDS-PAGE.

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 100°C for 5 min (reduced samples). Equal counts (polyclonal immunoprecipitates) or equal volumes (monoclonal immunoprecipitates) from each sample were loaded onto polyacrylamide gels and electrophoresis was carried out as previously described (21, 22). Fluorography was performed as previously described (4), and dried gels were exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.). Integrated optical densities of bands from nonenhanced autoradiographs were quantitated with a Millipore Bio-Image Visage 60 system. Autoradiographs of nonreducing 12.5% polyacrylamide gels were digitized with a Sharp JX-325 high-resolution scanner and enhanced by using Adobe Photoshop 2.5 software to increase the contrast between bands. All bands shown in Fig. 1A and Fig. 7 were enhanced equally.

RESULTS

Effect of DTT on E1-folding intermediates. It has been shown for several proteins that the formation of intramolecular disulfide bonds occurs both during and after translocation across the ER membrane (11, 13). Although little is known about the time of intramolecular disulfide bond formation (cotranslocational and/or posttranslocational) within Sindbis virus glycoprotein E1, these bonds are critical for the folding of E1, the assembly of the heterotrimeric spike, and the stability of the envelope protein lattice (2, 21, 23). We have previously shown that within the mature virus particle, E1 disulfides which stabilize the structural integrity of the envelope protein lattice are resistant to the reductive activity of DTT (2).

To determine at what point in its maturation E1 assumes a conformation which renders its disulfides more inaccessible to the reductive activity of DTT, we used a protocol employed in a previously published study examining the effect of short-term DTT treatment on the folding and oligomerization of the VSV G protein and influenza virus HA (33). Sindbis virus-infected BHK cells were pulse-labeled with [^{35}S]methionine-cysteine for 2 min and chased for specified times in the presence of cycloheximide. At the end of each chase, the monolayers were either lysed with detergent or chased for an additional 5 min in the presence of cycloheximide and 5 mM DTT prior to lysis. Quenching of DTT and the prevention of aberrant or incorrect disulfide bond formation in E1 during lysis were accomplished by washing the monolayers at 4°C in PBS containing the sulfhydryl-alkylating agent NEM (20 mM) and by including 20 mM NEM in the lysis buffer. Lysates were immunoprecipitated with polyclonal anti-E1 antiserum (21), and the immunoprecipitates were analyzed by SDS-PAGE under both nonreducing (Fig. 1A) and reducing conditions (6). The integral optical density for each of the individual E1 species isolated prior to or after DTT treatment for each chase time was calculated from densitometer tracings taken from autoradiographs. These values were converted to a percentage of the total E1 immunoprecipitated from each sample and are presented in Fig. 1B and C.

When Sindbis virus E1 was pulsed and chased without being subjected to the reductive activity of DTT, E1 was found to progress, under nonreducing conditions, from its more slowly migrating, less compact intermediates, E1α and E1β, into its faster-migrating intermediate, E1γ, and its stable and most compact conformation, E1ε (Fig. 1A [odd-numbered lanes] and B). E1ε is first seen at about 10 min after a 2-min pulse (Fig. 1A and B). When E1 is analyzed under reducing conditions, it migrates as a single band with a slightly slower mobility than that of E1α (6). These data are similar to the data previously published by Mulvey and Brown (21). However, when after the specified chase times E1 was chased for an additional 5 min in the presence of 5 mM DTT, the profile seen by nonreduced SDS-PAGE for E1 intermediates changed for each sample (Fig. 1A and C). Immediately after the pulse, approximately 90% of the E1 isolated was recovered as either completely reduced E1 or E1α, with a small amount of E1β and E1γ remaining after treatment with reducing agent (Fig. 1A [lane 2] and C). With increasing chase times prior to DTT treatment, the amount of E1β, E1γ, and E1ε present after treatment with reducing agent increased, and this increase coincided with a decrease in the amount of reduced E1 and E1α (Fig. 1A [lanes 4, 6, 8, and 10] and C). When monolayers were lysed in the absence of NEM prior to exposure to reducing agent, the E1 intermediates did not exhibit any sensitivity to DTT (6). This agrees with the findings of Tatu et al. (33)

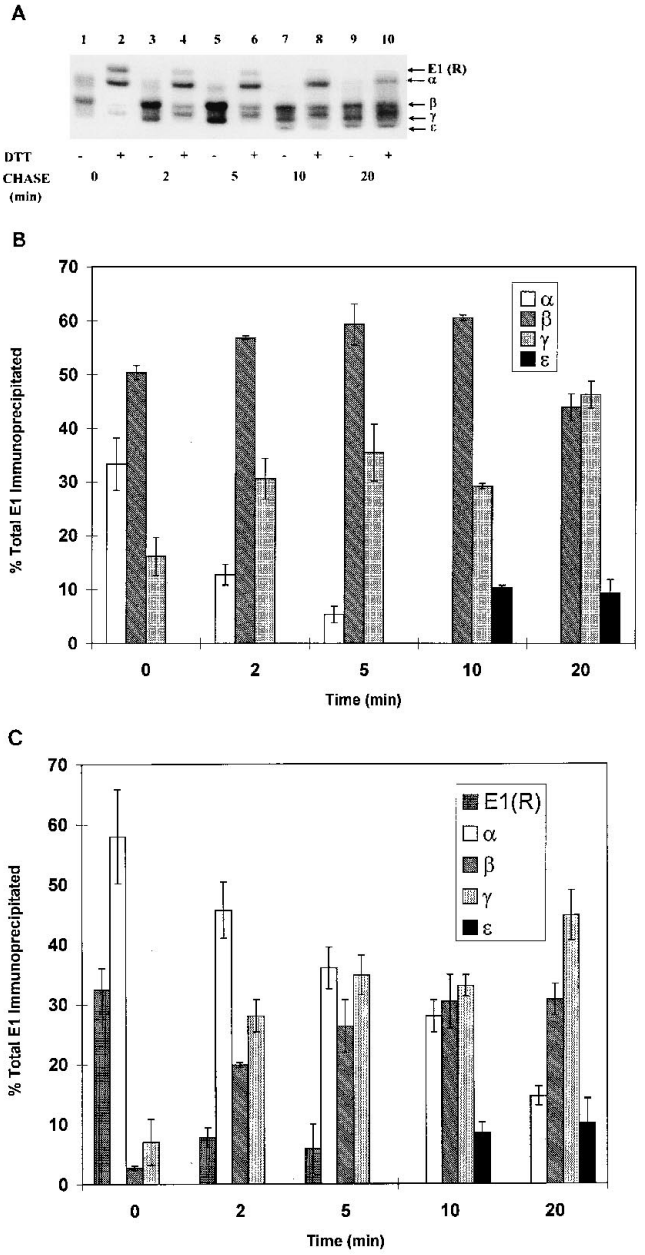

FIG. 1. Characterization of the accessibility of intramolecular disulfide bonds within the folding intermediates of Sindbis virus glycoprotein E1 to the reductive activity of DTT. (A) BHK cells infected with heat-resistant Sindbis virus were pulse-labeled for 2 min with [^{35}S]methionine-cysteine and then chased in the absence of DTT for the times shown. At the end of the chase, monolayers were either treated with NEM and lysed as described in Materials and Methods (−) or chased an additional 5 min in the presence of 5 mM DTT prior to NEM treatment and lysis (+). E1 was immunoprecipitated with polyclonal E1 antiserum, and equal counts were analyzed on nonreducing SDS–12.5% polyacrylamide gels. Autoradiographs were enhanced as described in Materials and Methods to increase the contrast between bands. The integrated optical densities of the individual E1 species resolved in the nonreducing gel (nonenhanced) were determined for three separate experiments and are presented as a percentage of the total E1 recovered at each chase time prior to (B) or after (C) a chase in DTT. E1(R), reduced E1; error bars, standard deviations.

that the effects of DTT on folding intermediates depend on the intact nature of the ER.

The absence of reduced E1 in pulse-chased cells not treated with DTT (Fig. 1A, lanes 1, 3, and 5) and the appearance of reduced E1 in the same cells after a 5-min chase in the presence of DTT (Fig. 1A [lanes 2, 4, and 6] and C) are evidence for intramolecular disulfide bond formation in E1 occurring cotranslationally as the polyprotein is translocated through the ER membrane. Also, our ability to isolate reduced E1 from the samples treated with reducing agent after a chase for 0, 2, and 5 min (Fig. 1A, lanes 2, 4, and 6) indicates that one or more of the E1 intermediates is sensitive to reduction by DTT. It is also significant that in each of the samples treated with DTT, E1 exists primarily as one or more of its three folding intermediates (Fig. 1A and C). This indicates that, unlike the folding intermediates of the VSV G protein or influenza virus HA, some of the E1 intermediates exhibit either partial or complete resistance to reduction by short-term DTT treatment. Finally, the mobilities of all the E1 species both before and after DTT treatment in nonreduced SDS-PAGE are the same, except that for E1γ, which has a slightly slower mobility after treatment with the reducing agent (Fig. 1A). The mobility of E1γ after exposure to DTT is similar to that of E1δ, an E1 species previously described by Mulvey and Brown (21) as a product of artifactual thiol-disulfide exchange reactions. It is possible that E1δ is a very transient folding intermediate which occurs as E1β folds into E1γ and is therefore not detectable in significant amounts by the methods described by Mulvey and Brown (21). Alternatively, this mobility change may be due to the sensitivity of one or more disulfides within E1γ to DTT. Since we are unable to differentiate between these two possibilities we will, for the sake of clarity, refer to both E1δ and E1γ as E1γ.

Resistance of E1γ and E1ε to DTT. A comparison of the amounts of E1γ and E1ε present before DTT treatment and the amounts present after exposure to reducing agent (Fig. 1B and C) confirms that both E1γ and E1ε are resistant to reduction by short-term DTT exposure. These data show that E1, unlike the G protein of VSV and HA of influenza virus, acquires a conformation which cannot be totally reduced by short-term DTT treatment prior to the completion of its folding into its native E1ε conformation. It follows therefore that neither E1γ nor E1ε contributes to the amount of reduced E1 isolated from the monolayers chased for 0, 2, and 5 min prior to DTT exposure (Fig. 1A).

Effect of DTT on E1α and E1β. Since E1γ and E1ε are not reduced by DTT, E1α and/or E1β is completely reduced upon exposure to DTT. Our ability to isolate reduced E1 only from those samples which contained E1α prior to DTT treatment (Fig. 1A [lanes 1 to 6] and C) suggested to us that E1α may have been the species which was completely reduced by exposure to reducing agent. The presence of E1α after DTT exposure could, then, be explained if E1β exhibited only partial sensitivity to DTT and was reduced no further than to a conformation with a mobility similar to that of E1α. Since we do not as yet have disulfide maps of any of the E1 species characterized by Mulvey and Brown (21), we cannot be certain that E1 isoforms which result from DTT treatment and exhibit the same mobility as an E1 intermediate isolated as part of the normal folding pathway of E1 contain the same number and/or arrangement of disulfide bonds. To determine if E1α was the E1 intermediate which was being reduced by exposure to DTT, for each sample we compared the amount of E1α present prior to treatment with DTT and the amount of reduced E1 present after exposure to the reducing agent (Fig. 2). As is shown in Fig. 2, the amount of E1α present prior to reduction is always similar to the amount of reduced E1 present after exposure to DTT. These data suggest that E1 which has folded no further than its α conformation remains completely sensitive to short-term DTT treatment.

The amount of E1β present prior to DTT exposure compared with the amount of E1α present after exposure to the reducing agent is significantly different (Fig. 3) from what is seen for the comparison of E1α and reduced E1 shown in Fig.

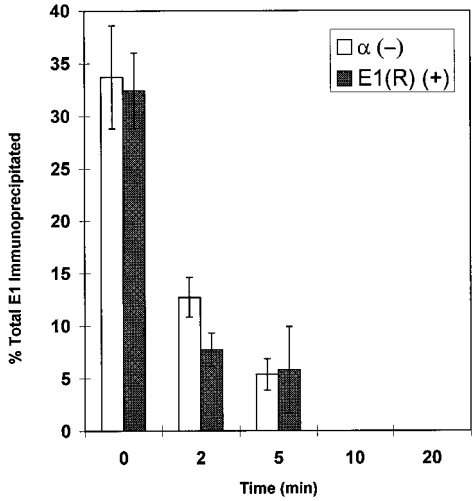

FIG. 2. E1α is completely reduced upon exposure to DTT. The integrated optical densities for E1α [α(−)] and reduced E1 [E1(R)] resolved for each time point (as presented in Fig. 1B and C) are graphed here to compare the amount of α(−) present prior to DTT treatment with the amount of E1(R) (+) present after treatment with DTT. Error bars, standard deviations.

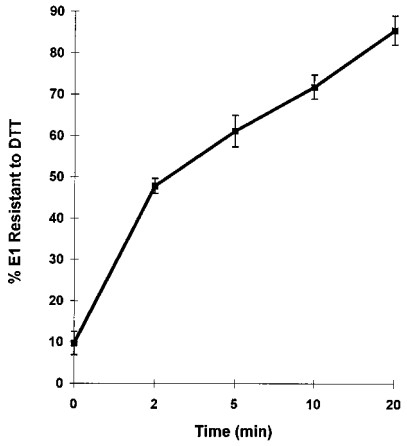

FIG. 4. E1 acquisition of resistance to short-term DTT exposure correlates temporally with BiP-E1 dissociation and oligomer formation. The amount of DTT-resistant E1 for each sample treated with DTT shown in Fig. 1A was plotted versus chase time as a percentage of the total amount of E1 isolated for each sample for three separate experiments. These values were graphed, and from this graph the $t_{1/2}$ for E1β acquisition of DTT resistance was calculated to be between 2 and 4 min. Error bars, standard deviations.

2. Initially, the E1 which has folded into the β conformation during the pulse appears to be returned to an α-like conformation upon exposure to DTT (Fig. 3). However, as the chase prior to DTT treatment was extended we observed an increase in the amount of E1β which was not reduced to E1α and was therefore exhibiting increased resistance to short-term DTT exposure (Fig. 3). This observation is further supported by the fact that the amount of E1β present prior to DTT treatment is always roughly equivalent to the amount of E1α and E1β present after exposure to DTT (Fig. 3). Together these data suggest that, after folding into its β conformation, E1 undergoes a transition from a DTT-sensitive state to a conformation which exhibits increased resistance to reduction by DTT. The fact that E1β isolated before or after the chase in DTT has the same mobility in nonreduced SDS-PAGE indicates that the transition into a DTT-resistant conformation does not coincide with any detectable change in the intramolecular disulfide bond arrangement of E1.

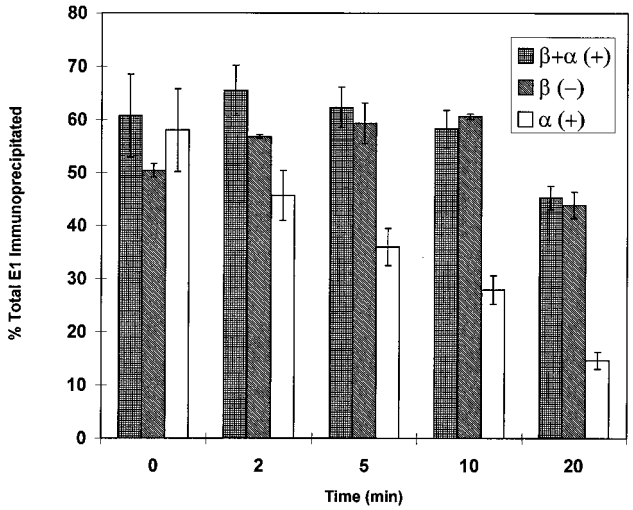

FIG. 3. The transition of E1 from a DTT-sensitive conformation into a conformation resistant to DTT occurs after E1 has folded into its E1β intermediate. The integrated optical densities for E1β and E1α resolved for each time point (as presented in Fig. 1B and C) are graphed here to compare the amount of E1β present prior to DTT treatment (−) with the amount of E1α and E1β present after treatment with DTT (+). Error bars, standard deviations.

Acquisition of resistance to DTT correlates temporally with E1 dissociation from BiP and oligomer formation. It has been proposed that ATP-dependent chaperones such as GRP78-BiP facilitate the reduction of folding intermediates by making hidden disulfides more accessible to reduction (33). In Sindbis virus-infected cells, the dissociation of BiP-E1 complexes occurs with a $t_{1/2}$ of ~4 min (22, 24). Formation of the PE2-E1 heterodimer occurs concurrently with or immediately preceding heterotrimer assembly with a $t_{1/2}$ of ~4 min (22, 23) and correlates with E1 folding into its E1β intermediate (5, 23). In order to determine if E1 acquisition of resistance to short-term DTT exposure could be temporally correlated with BiP-E1 dissociation and the formation of PE2-E1 oligomers, we calculated the $t_{1/2}$ for the formation of DTT-resistant E1. The $t_{1/2}$ of E1 acquisition of resistance to short-term DTT exposure was determined by plotting the amount of DTT-resistant E1 (the amount of E1β, E1γ, and E1ε present after exposure to the reducing agent) for each sample shown in Fig. 1A versus chase time. These values are presented in Fig. 4, and the $t_{1/2}$ for the formation of DTT-resistant E1 was calculated to be between 2 and 4 min. These data indicate that E1 acquisition of resistance to short-term DTT exposure is temporally correlated with the dissociation of BiP-E1 complexes, and like the formation of the VSV G protein (homotrimers) the acquisition of DTT resistance by Sindbis E1 occurs concurrently with oligomer (heterodimer and heterotrimer) formation in the ER.

Disulfide bonds within PE2 stabilize domains required for heterodimer formation. PE2 contains 19 cysteines which are highly conserved among all alphaviruses, 4 in E3, 12 in the lumenal domain of E2, and 3 in the E2 carboxyl tail (endodomain). Although we have been unable to detect any disulfide folding intermediates for PE2 (21), this does not rule out the possibility that intramolecular disulfides which are important for the maturation of the Sindbis virus spike exist within PE2. It is possible that PE2 contains disulfide bonds that upon their formation do not induce conformational changes which are

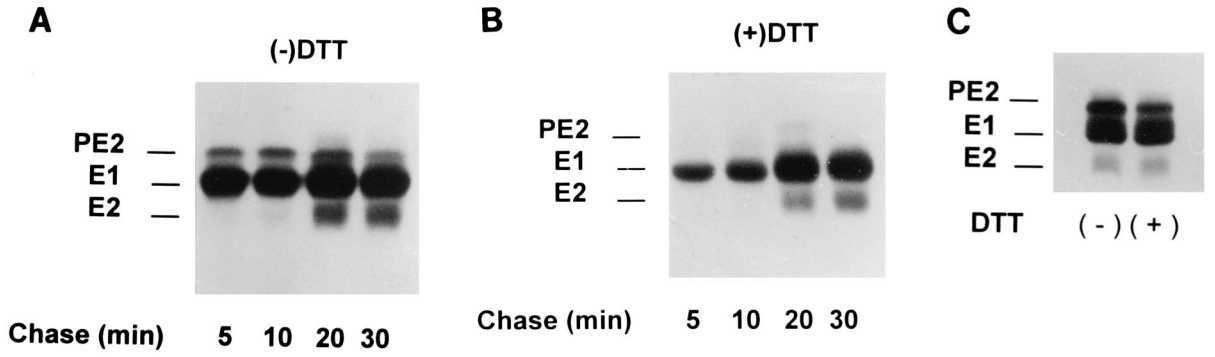

FIG. 5. Exposure of PE2-E1 heterotrimers in the ER to DTT disrupts PE2-E1 associations. Sindbis virus-infected BHK cells were pulse-labeled for 2 min and chased at 37°C as described in the legend to Fig. 1. At the end of the chase, infected monolayers were either treated with 20 mM NEM and lysed in the presence of NEM as described in Materials and Methods (−DTT) (A) or chased an additional 5 min in 5 mM DTT prior to NEM treatment and lysis (+DTT) (B). Lysates were immunoprecipitated with monoclonal E1 antiserum, and equal volumes of immunoprecipitated protein were analyzed by reducing 10.8% SDS-PAGE. Coprecipitation of PE2 with E1 monoclonal antiserum from cell lysates was used to assay for the presence of PE2-E1 heterodimers. (C) PE2-E1 associations in heterotrimers which have exited the ER are not sensitive to DTT. BHK cells infected with Sindbis virus were pulse-labeled for 5 min and chased for 2.5 h at 37°C in the presence of 25 μM monensin as previously described (5). At the end of the chase, one monolayer was washed and lysed in the presence of 20 mM NEM (−) and the other was chased an additional 5 min in the presence of 5 mM DTT prior to NEM treatment and lysis (+) as described in Materials and Methods. Lysates were immunoprecipitated with monoclonal E1 antiserum, and immunoprecipitated proteins were analyzed by reducing 10.8% SDS-PAGE. Coprecipitation of PE2 with E1 monoclonal antiserum from cell lysates was used to assay for the presence of PE2-E1 heterodimers.

extensive enough to be detectable under the conditions which we have used to characterize the folding intermediates of E1 (21). We have previously shown that E1-E1 associations residing in the mature spike stabilize the envelope protein lattice and are themselves stabilized by intramolecular disulfide bonds (1, 2).

During our analysis of the effect of DTT on E1-folding intermediates we determined that at the times postsynthesis at which E1 exhibited resistance to short-term DTT exposure, the association of PE2 with E1 remained sensitive to DTT (Fig. 5A and B). This was determined by comparing the amount of PE2-E2 which could be coprecipitated with E1 monoclonal antiserum from lysates of Sindbis virus-infected cells which were pulse-labeled and chased as described in the legend to Fig. 1. As is indicated by the data in Fig. 5A and B, PE2-E1 associations are very sensitive to disruption by DTT while E1-E2 associations are resistant to such disruption. The decrease in the amount of E1 immunoprecipitated from cells treated with DTT after a chase of 5 or 10 min (Fig. 5B) is due to our monoclonal E1 antiserum's being conformation specific and not precipitating either E1α or reduced E1 (6) which is present at these times after DTT exposure (Fig. 1A). If however Sindbis virus-infected cells are pulse-labeled and chased for 2.5 h in the presence of the carboxylic ionophore monensin in order to export and trap the majority of pulse-labeled PE2-E1 heterotrimers in the medial Golgi apparatus (5), subsequent treatment with DTT does not significantly affect PE2-E1 associations (Fig. 5C) and E1 remains resistant to reduction (6). This observation taken together with the observation that E1-E2 associations are also resistant to DTT (Fig. 5B) confirms the observations of Tatu et al. (33) that short-term effects of DTT treatment on whole cells are limited to newly synthesized proteins and folding intermediates in the ER.

The data above suggest that PE2 contains disulfide bonds which are required for the stability of domains involved in PE2-E1 associations and heterodimer formation. When lysates from Sindbis virus-infected cells prepared as described in the legend to Fig. 6 are immunoprecipitated with whole-virus polyclonal antiserum and analyzed on a 4 to 10% gradient SDS-polyacrylamide gel under nonreducing conditions, we are able to detect a mobility change in the PE2 protein after exposure to DTT (Fig. 6). PE2 from the samples shown in Fig. 7 exhibit the same mobility when analyzed under reducing conditions, thus indicating the presence of disulfide bonds within PE2 (6). The decrease from the amount of PE2 precipitated from untreated cells to that from DTT-treated cells (Fig. 6) is because of the lower level of efficiency of our whole-virus antiserum at precipitating reduced PE2 (6). It is also important that the different E1 isoforms are not resolvable under the PAGE conditions described in the legend to Fig. 6.

Together these data demonstrate that PE2 does contain intramolecular disulfide bonds and that these bonds are important for stabilizing domains required for heterodimer formation. These data also show that the disulfides in ER-resident PE2 which are required for the stabilization of domains involved in heterodimer formation continue to be accessible to the reductive activity of DTT. We speculate that the continued sensitivity of PE2 intramolecular disulfides to DTT results from the existence of PE2 in a conformation more extended than that of E1 and/or the positioning of PE2 disulfide-con-

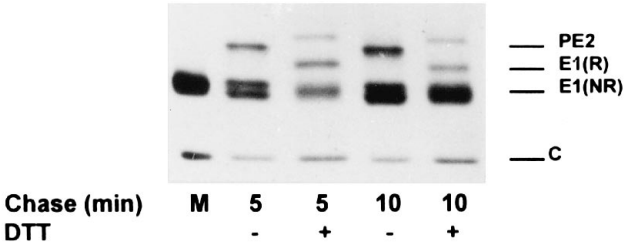

FIG. 6. PE2 contains intramolecular disulfide bonds. Sindbis virus-infected BHK cells were pulse-labeled for 5 min and chased for the specified times at 37°C as described in Materials and Methods. At the end of each chase one monolayer was washed and lysed in the presence of 20 mM NEM (−) and the other was chased an additional 5 min in the presence of 5 mM DTT prior to NEM treatment and lysis (+) as described in Materials and Methods. Lysates were immunoprecipitated with polyclonal whole virus antiserum, and immunoprecipitated proteins were analyzed by SDS-PAGE on a 4 to 10% nonreducing gradient. A decrease in the mobility of PE2 after exposure to DTT indicates the presence of disulfide bonds within PE2. M, nonreduced ^{35}S-labeled whole-virus marker (E1 and E2 migrate as one band under these gel conditions); E1(R), reduced E1; E1(NR), E1 which exists as either E1α, E1β, E1γ, or E1ε (the nonreduced E1-folding intermediates and the fully folded E1ε conformation are not separated under these gel conditions).

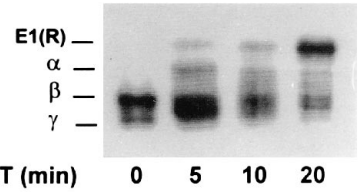

FIG. 7. Continued exposure of E1 to DTT after dissociation of PE2-E1 complexes results in an increase in E1 sensitivity to DTT. Sindbis virus-infected BHK cells were pulse-labeled for 5 min and chased for 5 min at 37°C as described in Materials and Methods. At the end of each chase, one monolayer was washed and lysed in the presence of 20 mM NEM and the other monolayers were chased an additional 5, 10, or 20 min in the presence of 5 mM DTT prior to NEM treatment and lysis as described in Materials and Methods. E1 was immunoprecipitated with polyclonal E1 antiserum, and equal counts were analyzed on nonreducing 12.5% polyacrylamide gels. Autoradiographs were enhanced as described in Materials and Methods to increase the contrast between bands.

taining domains at a more accessible location(s) at the periphery of the spike.

Extended treatment with DTT increases E1 sensitivity to reduction. We have previously shown that the structural integrity of the Sindbis virus envelope is dependent upon E1-E1 associations stabilized by intramolecular disulfide bonds (1, 2). Disruption of these bonds in the mature virus particle requires either extended treatments with DTT (3 h or more) or the induction of significant conformational changes (low-pH treatment) prior to exposure to DTT (2). If the structural domains stabilized by E1 disulfides are buried within the spike, as we have proposed (1, 2), then their inaccessibility to DTT could be mediated by both the fully folded native E1 conformation and the positioning of E2 in the heterotrimeric spike complex.

The data presented above show that DTT treatment of Sindbis virus-infected cells can dissociate PE2 from E1 at times when heterotrimer formation has occurred (23) and E1 has acquired resistance to short-term DTT exposure. We have tested the role of PE2 in E1 acquisition of resistance to short-term DTT exposure by analyzing the sensitivity of the more compact E1 species to extended treatments with DTT after PE2-E1 dissociation. Sindbis virus-infected cells were pulse-labeled for 5 min and chased for 5 min as described in Materials and Methods. One monolayer was lysed after the chase, while three other monolayers were chased an additional 5, 10, and 20 min in the presence of 5 mM DTT prior to lysis. All monolayers were washed and lysed in the presence of 20 mM NEM as previously described (5, 21). Lysates were immunoprecipitated with polyclonal E1 antiserum, and immunoprecipitated protein was analyzed by 12.5% nonreducing PAGE (Fig. 7).

The data presented in Fig. 7 indicate that exposure of Sindbis virus-infected cells to DTT for times in excess of that required to dissociate PE2 from E1 (5 min) (Fig. 5) results in an increase in the amount of E1 which is reduced by DTT as well as an increase in the sensitivity of E1 isoforms E1β and E1γ to DTT. These data indicate that the presence of PE2 in the heterotrimer appears to play a role in mediating the inaccessibility of disulfides within E1-folding intermediates to DTT. We speculate that once the reduction of PE2 disulfide(s) occurs and PE2-E1 associations are disrupted (disassembly of the spike), E1 disulfides become more accessible to the reductive activity of DTT. This is further supported by evidence that E1 expressed in the absence of PE2 is able to fold into compact conformations yet remains completely sensitive to reduction when subjected to short-term DTT exposure (7).

DISCUSSION

Viral envelope glycoproteins, including Sindbis virus glycoproteins, are excellent models for the study of cotranslational and posttranslational protein folding within the ER. As the nascent Sindbis virus E1 protein folds, its glycosylation, interaction with GRP78-BiP, and formation of intramolecular disulfide bonds are all ER-resident events which likely drive the formation of specific folding intermediates and thereby facilitate the folding process (11, 21, 22, 29). Most if not all viral envelope glycoproteins contain one or more structural domains which, prior to the formation of quaternary associations required for oligomer assembly, must first fold into their proper tertiary conformation (11, 15). The structural integrity of the Sindbis virus envelope protein lattice is dependent upon E1-E1 interactions which are stabilized by intramolecular disulfide bonds (1, 2). The inability of the reducing agent DTT to significantly disrupt the structure of the envelope protein lattice strongly suggests that the E1 intramolecular disulfide bonds which stabilize the protein lattice are buried deep within structural domains in the native conformation of the heterotrimers and become readily accessible to DTT only after the induction of significant conformational changes (2).

We have previously shown that Sindbis virus glycoprotein E1 transiently associates with GRP78-BiP, folds through a series of unstable disulfide intermediates which differ in the number and/or arrangement of their intramolecular disulfide bonds, and is assembled into heterotrimers prior to assuming its native and most compact conformation (E1ε) (7, 21–23). Through pulse-chase analysis we have characterized the relative accessibility of intramolecular disulfide bonds within Sindbis virus glycoprotein PE2 and the folding intermediates of Sindbis virus glycoprotein E1 to the reductive activity of DTT. The data presented above suggest that intramolecular disulfide bond formation within E1 occurs cotranslationally, as we are only able to isolate reduced E1 from pulse-labeled Sindbis virus-infected cells treated with DTT prior to lysis. We have shown that E1α is the only intermediate totally reduced by short-term exposure to DTT. Our results demonstrate that acquisition of increased resistance to DTT occurs after E1 has folded into its β conformation, and this correlates temporally with the dissociation of BiP-E1 complexes and with oligomer (heterotrimer) formation (22–24). Our observation that the transition of E1β from a DTT-sensitive state into a DTT-resistant state is not accompanied by a detectable change in the mobility of E1β, analyzed by nonreducing SDS-PAGE, suggests that the acquisition of increased resistance to DTT by E1 does not result from a specific disulfide bond formation or isomerization event within E1.

We have also demonstrated that intramolecular disulfide bonds exist within PE2. PE2 disulfides are sensitive to DTT within the ER, and disruption of these disulfides leads to dissociation of PE2-E1 heterodimers. Once PE2 is no longer associated with E1, ER-resident E1-folding intermediates that had previously shown resistance to short-term DTT exposure are reduced upon extended DTT treatment. These data indicate that disulfides within PE2 are required for the stabilization of domains necessary for the formation of PE2-E1 heterodimers and remain accessible to the reductive activity of DTT even after heterotrimer formation. The disulfide-stabilized PE2 domains may also be positioned within the heterotrimer in a conformation which increases the inaccessibility of E1 disulfides to DTT.

Our results confirm the observations of Tatu et al. (33) that the effect of short-term DTT treatment on disulfide bonds within proteins moving through the secretory pathway is lim-

ited to newly synthesized proteins and folding intermediates in the ER. It has been proposed that DTT can act indirectly upon proteins containing disulfide bonds by reducing the number of active-site cysteines in protein disulfide isomerase, thereby activating its reductase activity (33). Since protein disulfide isomerase is an ER-resident protein, this could explain the seemingly organelle-specific nature of DTT's reductive activity and could also explain why short-term DTT treatment does not disrupt the structural integrity of the mature Sindbis virus envelope (2). Both E1 and E2 are present in the Sindbis virus envelope as fully folded, native proteins contained in the spike. Eighty of these spikes are organized into a T=4 icosahedral protein lattice via lateral associations between spikes and capsid-E2 interactions which further stabilize the integrity of the envelope lattice. In the ER, DTT may be more capable of spike disassembly because of its possible indirect effect on protein disulfide isomerase and/or the structural differences which exist between a PE2-E1 heterotrimer and an E1-E2 heterotrimer incorporated into a highly constrained and well-ordered icosahedral protein lattice.

Exposure of mature Sindbis virus to DTT for periods of time which do not disrupt the structural domains of the envelope protein lattice do however significantly disrupt the functional domains, resulting in virus inactivation (2). Glycoprotein E2 has been shown to be important in the mediation of virus-cell attachment (20, 30–32, 35–37). The presence of PE2 intramolecular disulfide bonds which remain accessible to the reductive activity of DTT after spike trimer formation in the ER could indicate that it is the reduction of disulfide bonds within E2 which results in virus inactivation by preventing virus-cell attachment. This possibility is presently under investigation.

The inaccessibility of E1 disulfides, responsible for the structure of the mature virus spike, to DTT (2) and the formation of the heterotrimer concurrently with or soon after E1's folding into its E1β conformation (23) together with the data presented above argue strongly that the event which mediates the increased inaccessibility of E1 disulfides to DTT is heterotrimer formation. We propose that upon heterotrimer assembly in the ER, E1 disulfides which are required for the structural integrity of the Sindbis virus envelope protein lattice are buried within the spike complex and are in part inaccessible to DTT because of the surrounding PE2-E2 domains. Our results support those of previously published studies which investigated the structure of the Sindbis virus spike and the orientations of E1 and E2 domains (1, 30).

ACKNOWLEDGMENTS

This research was supported by grant AI14710 from the National Institutes of Health and by the Foundation for Research, Carson City, Nev.

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