Formation and Rearrangement of Disulfide Bonds during Maturation of the Sindbis Virus E1 Glycoprotein

MATTHEW MULVEY AND DENNIS T. BROWN*

*Cell Research Institute and Department of Microbiology, University of Texas
at Austin, Austin, Texas 78713-7640*

Received 1 July 1993/Accepted 2 November 1993

The rigidly ordered icosahedral lattice of the Sindbis virus envelope is composed of a host-derived membrane bilayer in which the viral glycoproteins E1 and E2 reside. E1-E1 interactions stabilized by intramolecular disulfide bridges play a significant role in maintaining the envelope's structural integrity (R. P. Anthony and D. T. Brown, *J. Virol.* 65:1187–1194, 1991; R. P. Anthony, A. M. Paredes, and D. T. Brown, *Virology* 190:330–336, 1992). We have examined the acquisition of disulfide bridges within E1 during its maturation. Prior to exit from the endoplasmic reticulum, E1 folds via at least three intermediates, differing in the number and/or arrangement of their disulfides, into a single, compact form. This E1 species remains stable with respect to its disulfides until late in the secretory pathway, when E1 attains a metastable conformation. At this point, when appropriately triggered, intramolecular thiol-disulfide exchange reactions within E1 can occur, resulting in the generation of alternative E1 species. This metastable nature of mature E1 may have important implications for the mechanism of virus disassembly during the initial stages of the infection process (B. Abell and D. T. Brown, *J. Virol.* 67:5496–5501, 1993).

Sindbis virus is a plus stranded RNA virus adept at utilizing both vertebrate and invertebrate cellular machinery for its own replication (6). The mature virion possesses a highly structured envelope composed of a host-derived membrane bilayer in which reside 240 copies each of the viral glycoproteins E1 and E2 (2). The COOH-terminal tail of each E2 penetrates the envelope and interacts with the underlying nucleocapsid, which contains the genomic RNA associated with 240 copies of a basic capsid protein (C). Both the envelope and the nucleocapsid have T=4 icosahedral symmetry (33).

The virus structural proteins are synthesized as a large polyprotein from a subgenomic RNA in the order NH₂-C-PE2-6K-E1-COOH (39). The capsid is autoproteolytically cleaved from the developing nascent chain and rapidly incorporated into the nucleocapsid. The remaining polypeptide is directed by the signal recognition particle into the rough endoplasmic reticulum (ER), where it is cotranslationally cleaved and glycosylated (4, 39). PE2 and E1, which are type III and type I membrane proteins, respectively, are transported as a noncovalently associated hetero-oligomeric complex through the cell's secretory pathway (36, 44). Late in this pathway, PE2 is processed to produce E2 and a small glycopeptide, E3, that is secreted into the medium. At the plasma membrane, the association of E2 tails with nucleocapsids is thought to initiate a budding process which leads to the release of mature virions (27–29).

During their maturation, E1 and E2 must be driven towards specific tertiary and quaternary conformations which not only allow them to be successfully assembled into virions but also allow for disassembly of the virion upon interaction with a host cell. The Sindbis virion envelope contains heterodimers of E1 and E2 arranged into eighty tightly complexed trimers, which appear as spikes on the virus surface (2, 33). These spikes have rigid lateral associations with one another throughout the

envelope (33). It has been demonstrated that the envelope's rigid structure is maintained by E1-E1 interactions stabilized by intramolecular disulfide bridges buried within the spike protein complexes (2, 3). Upon interaction of the virus with an appropriate cell surface receptor during the initial stages of infection, conformational changes are induced within the Sindbis envelope proteins (17, 30). Workers in our laboratory have proposed that such conformational changes may allow a rapid reshuffling of disulfide bridges via thiol-disulfide exchange reactions within the Sindbis virus E1 glycoprotein (1, 3). These disulfide rearrangements could result in disruption of the protein-protein interactions which stabilize the rigid protein lattice of the envelope, enabling the viral envelope to fuse with a cellular membrane. This model implies that E1 is a metastable structure and emphasizes the critical importance of the arrangement of disulfide bonds within E1 during both assembly and disassembly of the Sindbis envelope.

E1 contains 16 cysteine residues within its ectodomain which are highly conserved among the alphaviruses (40); however, the number and arrangement of the disulfides within E1 have yet to be determined. The formation of disulfide bridges within transmembrane and secretory proteins typically occurs within the ER during or soon after translocation and is an intrinsic part of the folding process (18). The folding of many proteins proceeds via intermediates which differ from their mature native structures in the configurations of their disulfide bridges (5, 12, 15, 20, 22, 31, 34, 41). In this study, the formation and rearrangement of disulfides within E1 during its maturation have been investigated. Evidence is presented supporting the hypothesis that the mature form of E1 is metastable with respect to the arrangement of its disulfide bridges.

MATERIALS AND METHODS

Cells and virus. Baby hamster kidney (BHK-21) cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum (GIBCO), 5% tryptose phosphate broth, and 2 mM L-glutamine. Stocks of the heat-resistant

* Corresponding author. Mailing address: Cell Research Institute, University of Texas at Austin, Austin, TX 78713-7460. Phone: (512) 471-1431. Fax: (512) 471-3353.

SVHR strain of Sindbis virus, derived originally by E. R. Pfefferkorn, were produced as described previously (35).

Infection, drug treatment, and metabolic labeling of cells. Subconfluent monolayers were treated at 37°C with medium containing dactinomycin (4 µg/ml; Calbiochem) for 60 min before infection with ~100 PFU of SVHR per cell. Infected monolayers were incubated at 37°C in medium containing dactinomycin for 4 h and then starved for 10 to 20 min in methionine- and cysteine-free medium supplemented with 1% fetal calf serum. The cells were pulsed at 37°C with 50 µCi of [³⁵S]methionine plus [³⁵S]cysteine ([³⁵S]methionine/cysteine) (New England Nuclear) per ml and chased in medium containing 1 mM nonradioactive methionine and 75 µg of cycloheximide per ml. All media throughout each experiment contained 2 mM gentamicin.

Where indicated, cells were treated with 20 µg of nocodazole (Janssen Biotech) and/or 5 µg of brefeldin A (BFA; Epicentre Technologies) per ml. A 5-mg/ml stock solution of BFA in methanol was stored at -20°C. Nocodazole was freshly prepared before use.

Immunoprecipitation of E1. After the pulse-chases, monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) containing cycloheximide and then, except where noted, incubated on ice with 20 mM *N*-ethylmaleimide (NEM) in PBS for 10 to 20 min. Each monolayer was lysed on ice in 1 ml of lysis buffer (pH 7.2), containing 1% Triton X-100, 0.02 M Tris-HCl, 3.9 mM Tris base, 0.15 M NaCl, 0.5% deoxycholate, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (Sigma), 0.2 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (Sigma), and, except where stated otherwise, 20 mM NEM. The nuclei and large cell debris were pelleted and discarded.

Antibodies against E1 were isolated from rabbit antiserum by using a 1-ml HiTrap protein A column (Pharmacia) and then conjugated to CNBr-activated Sepharose beads (Sigma). Each lysate was treated overnight at 4°C with 200 µl of a 10% suspension of beads in lysis buffer. The bead-protein complexes were then washed three times with lysis buffer and resuspended in 30 µl of 2× nonreducing sample buffer (4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.02% bromophenol blue, 0.125 M Tris-HCl [pH 6.8]). After incubation at 80°C for 4 min, aliquots of each sample were mixed 1:1 with 2× sample buffer containing 10% 2-mercaptoethanol and heated for an additional 4 min at 100°C.

Immunoprecipitated proteins were digested with endoglycosidase H (endo H; Boehringer Mannheim) by protocols similar to those described previously (24).

Virus purification. [³⁵S]methionine/cysteine-labeled SVHR virions were purified by methods similar to those described previously (2). At 30 h postinfection, virus from the medium of radiolabeled infected cells was purified by density gradient centrifugation three times through 15 to 35% potassium tartrate gradients. Virus bands were dialyzed against PBS deficient in calcium and magnesium.

PAGE. ³⁵S-labeled proteins from immunoprecipitates or purified virus in reducing or nonreducing sample buffer were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (37). Samples were loaded onto a 3% acrylamide stacking gel, and the proteins were resolved in 12.5% acrylamide. Electrophoresis was carried out at a constant power of 5 W for 7.5 h (or 6 h where noted). Gels were impregnated with 2,5-diphenyloxazole (4a), dried, and exposed to X-Omat AR X-ray film (Eastman Kodak, Rochester, N.Y.). Integrated optical densities of bands from the autoradiographs were quantitated with a Millipore Bio-Image Visage 60 system. Autoradiographs of nonreducing gels were digitized and then

enhanced by using NIH Image 1.44 software (Wayne Rasband, National Institutes of Health [NIH] Research Services Branch, National Institute of Mental Health) to increase contrast between bands.

RESULTS

E1 can assume multiple disulfide-bonded conformations.

To investigate the involvement of disulfides in the folding pathway of the Sindbis virus E1 glycoprotein, BHK cells were pulse-labeled at 4 h postinfection with SVHR for 2 min with [³⁵S]methionine/cysteine and chased for various times with cycloheximide and excess nonradioactive methionine. The monolayers were then washed, incubated on ice with a 20 mM concentration of the membrane-permeable alkylating agent NEM (to block free thiols and inhibit artifactual disulfide formation or rearrangements [20, 34]), and then lysed in buffer containing NEM. E1 was immunoprecipitated from the lysates with monospecific polyclonal antibodies and analyzed by SDS-PAGE under reducing and nonreducing conditions. In the reducing gel (Fig. 1A), E1 migrates as a single band at all chase times. In the nonreducing gel (Fig. 1B), E1 is resolved into multiple species which differ in the number and/or arrangement of their disulfide bridges. Immediately after the pulse, four species of E1 were detected and designated E1α, -β, -δ, and -γ. E1α has an electrophoretic mobility similar to that of fully reduced E1. A fifth E1 species, E1ε, appears by 10 min of chase, and a sixth and final species, E1ζ, is seen below E1β by 30 min of chase. Between 30 and 45 min of chase, the mobility of E1ε appears to be decreased, bringing it closer to E1γ.

Unlike most other proteins which have been examined (5, 12, 15, 20, 31, 34, 41), E1 does not appear to be chased into a single, final disulfide-bonded form, as judged from the results in Fig. 1B (summarized in Fig. 1C). Instead, multiple E1 species that vary in the number and/or arrangement of their disulfide bonds are recovered even after long chases. The significance of this finding was investigated further.

Export of E1 from the ER. Typically, the correct folding and oligomerization of membrane and secretory proteins are prerequisites for the export of these proteins from the ER (18, 21). Assuming that similar restrictions apply to E1, incompletely folded or misfolded E1 species are expected to be retained within the ER. The acquisition of resistance to the enzyme endo H can be used to determine whether a protein has attained a transport-competent conformation and has left the ER and reached the medial Golgi stacks (24). Figure 2 shows the results of endo H assays performed on the total E1 immunoprecipitated from pulse-chased, infected BHK monolayers. Within 17 min after a 2-min pulse, 50% of the total E1 becomes endo H resistant. Approximately 81% of the E1 becomes endo H resistant after 45 min of chase time.

These results indicate that the export of E1 from the ER and processing into an endo H-resistant form is asynchronous, and this could reflect, in part, asynchrony in the acquisition of correct, disulfide-stabilized conformations by the maturing E1 molecules. Such asynchrony in folding could account for the existence of multiple disulfide-bonded E1 species (folding intermediates) seen after the shorter chase times (Fig. 1B). However, asynchrony in folding seems to be an inadequate explanation for the heterogeneity of the E1 species seen after the longer chase periods. By 45 min of chase in Fig. 1B, the majority of the nonreduced E1 species recovered are endo H resistant and thus represent correctly folded protein which has been exported from the ER. These E1 isoforms could be either (i) multiple, differentially disulfide-bonded, transport-competent E1 species or (ii) products of disulfide rearrangements

FIG. 1. Pulse-chase analysis of the Sindbis virus E1 glycoprotein. BHK cells infected with SVHR were pulse-labeled for 2 min with [35 S]methionine/cysteine and then chased for various times. Monolayers were treated with NEM before and during lysis as described in Materials and Methods. E1 was immunoprecipitated and analyzed by SDS-PAGE under reducing (A) and nonreducing (B) conditions. (C) The integrated optical densities of the individual E1 species resolved in the nonreducing gel (nonenhanced) were determined and are presented as a percentage of the total E1 recovered at each chase time. The electrophoretic mobility of E1 is faster than that of E2 under nonreducing conditions and slower under reducing conditions (43).

triggered during the process of isolation within one transport-competent E1 species which has progressed beyond the ER. The former possibility seems unlikely and would be unprecedented if true. The second possibility implies that, prior to exit from the ER, E1 has assumed a single conformation which is or becomes, later in the secretory pathway, unstable with respect to the arrangement of its disulfide bonds and inaccessible to NEM.

E1 folding within the ER. To determine whether E1 is folded into a single, disulfide-bonded conformation prior to export from the ER, the transport of E1 to the Golgi stacks was inhibited by a protocol employing the microtubule-disrupting agent nocodazole and the fungal metabolite BFA (26). Nocodazole inhibits the microtubule-dependent retrograde transport into the ER of the early compartments of the Golgi apparatus, which contain the enzymes responsible for acquisition of endo H resistance. BFA induces the microtubule-

dependent redistribution of the Golgi into the ER and inhibits the export of proteins from the ER. In combination, these two agents can effectively isolate the ER from the later compartments of the cell's secretory pathway (26).

To restrict E1 maturation to those events occurring in the ER, infected BHK cells at 1 h postinfection were incubated with nocodazole for 2 h at 37°C and then treated for an additional hour at 37°C with BFA plus nocodazole. Cells were then pulse-labeled for 5 min with [35 S]methionine/cysteine and chased for various times (as described in Materials and Methods). Nocodazole and BFA were maintained in the medium throughout the pulse-chases. As before, the cells were then treated with NEM on ice and lysed, and E1 was immunoprecipitated and analyzed by reducing and nonreducing SDS-PAGE. Under reducing conditions (Fig. 3A, lanes 1 to 4), E1 migrates as a single band. The mobility of reduced E1 increases slightly as the chase time progresses, probably because of

FIG. 2. Acquisition of endo H resistance by the Sindbis virus E1 glycoprotein. SVHR-infected BHK cells were pulse-labeled for 2 min and then chased for 0, 10, 20, or 45 min. Cells were treated with NEM and lysed, and E1 was immunoprecipitated as described in the legend to Fig. 1. The immunoprecipitates were divided and incubated with or without endo H for 16 h at 37°C as described in Materials and Methods. The samples were then electrophoresed under reducing conditions, and the percentages of the total E1 which were endo H sensitive and resistant at each chase time were quantitated as described in the legend to Fig. 1C.

FIG. 3. E1 folding and processing in nocodazole- and BFA-treated cells. Where indicated, SVHR-infected BHK monolayers were treated with nocodazole alone, BFA alone, nocodazole plus BFA, or untreated. Nocodazole treatment was initiated at 1 h postinfection, and BFA treatment began at 3 h postinfection. Cells were pulse-labeled for 5 min after a brief starvation period at 4 h postinfection and then chased for the times indicated. Nocodazole and/or BFA was maintained in the medium throughout the starvation period and the pulse-chases. In lane 11 (A and B), the BFA-treated cells, after a 5-h chase, were washed three times with warm PBS plus cycloheximide and then chased for an additional hour in the absence of BFA. After the pulse-chases, except where indicated otherwise, the cells were incubated and then lysed in the presence of NEM. E1 was immunoprecipitated from the lysates and analyzed by SDS-PAGE under reducing (A) and nonreducing (B) conditions. Alternatively, immunoprecipitates were divided and incubated with or without endo H as in Fig. 2. These samples were analyzed by electrophoresis for 6 h under reducing conditions (C), allowing a small amount of capsid protein (C, not shown), which is probably nonspecifically coprecipitated with E1 and which is of constant molecular weight, to remain in the gel. The distance between endo H-treated E1 (+) and C as a percentage (R) of the distance between non-endo H-treated E1 (–) and C was determined (24) and indicates the degree of resistance to endo H acquired by E1 under the various conditions.

processing of the two oligosaccharide side chains of E1 by ER-resident glycosidases (39). Under nonreducing conditions (Fig. 3B, lanes 1 to 4), E1 appears to progress through at least three intermediate forms, E1 α , β , and γ , into a fast-migrating species resembling E1 ϵ . After 2 h of chase, E1 remains endo H sensitive (Fig. 3C), and almost all of E1 has folded into the E1 ϵ species. This species is stable even when treatment with NEM is omitted from the experimental protocol (Fig. 3B, lane 5). These data demonstrate that E1 can attain a single, fast-migrating conformation prior to export from the ER. Additional data which show that some E1 can fold, although inefficiently, into E1 ϵ when transport from the ER is blocked by depleting cellular ATP levels (9, 27) confirm that E1 can complete folding into E1 ϵ within the ER (unpublished observation). This result also suggests a role for ATP in the efficient folding of E1 within the ER, as has been indicated for the folding of other proteins (13).

Post-ER/Golgi conformational change in E1. When E1 is chased for 2 h in non-drug-treated cells or in cells treated with only nocodazole (Fig. 3B, lanes 6 and 7), multiple disulfide-bonded E1 species, similar to those seen after the longer chases in Fig. 1B, are recovered instead of the single E1 ϵ form. These E1 species are primarily endo H resistant (Fig. 3C). Additionally, their electrophoretic mobilities under reducing conditions (Fig. 3A, lanes 6 and 7), compared with that of E1 which has not been exported from the ER, are decreased, probably because of the addition of complex sugars to E1 later in the secretory pathway (39). These results, along with those presented above, suggest that a conformational change in E1 ϵ occurs at some point beyond the ER which allows disulfides within this species to rearrange when triggered. An indication of where, more specifically, this conformational change in E1 ϵ might occur is provided when chases are performed with cells treated with only BFA, allowing the

return of Golgi-associated enzymes to the ER (23). During a 2-h chase of BFA-treated cells, most of E1 folds into the E1 ϵ species (Fig. 3B, lane 8), similarly to E1 in cells treated with both nocodazole and BFA (Fig. 3B, lane 4). E1 is only slightly endo H resistant by 2 h of chase in BFA-treated cells despite the presence of Golgi enzymes (Fig. 3C). E1 becomes more endo H resistant by 5 h of chase in cells treated with BFA. It has been shown that vesicular stomatitis virus G protein folds and oligomerizes at apparently normal rates in BFA-treated cells but becomes endo H resistant at a slower rate under the effects of this agent (14). In this respect, the processing of E1 to an endo H-resistant form in BFA-treated cells is similar to that reported for vesicular stomatitis virus G protein. After the extended 5-h chase of cells treated with BFA, most of E1 is still recovered in the E1 ϵ conformation, which remains stable even in the absence of treatment with NEM (Fig. 3B, lanes 9 and 10). When BFA is washed from the cells after the 5-h chase and the cells are then chased for an additional hour in the absence of BFA, multiple diffuse E1 bands are recovered with mobilities similar to those of the E1 species seen after the longer chases in Fig. 1B (Fig. 3B, lane 11).

Although BFA induces the return of the *cis*, medial, and *trans* components of the Golgi to the ER, it does not have this effect on the later compartments of the secretory pathway, including the trans-Golgi network and the endocytic pathway (14, 23). These results therefore suggest that E1, after folding into the E1 ϵ species, can remain stable with respect to its disulfide bonds after processing by both ER and Golgi enzymes. Furthermore, they imply that, at a post-Golgi location, E1 ϵ assumes a conformation in which its disulfides can be rearranged. These rearrangements are most likely initiated during or after cell lysis. The alternative possibility, that E1 folds into a single, fast-migrating conformation within the ER and then unfolds into multiple species late in the secretory pathway, seems improbable.

E1 recovered from Sindbis virions. If the intracellular end product of E1 maturation is the E1 ϵ species, which is able to rearrange into multiple conformations when appropriately triggered, the E1 present in mature virions should also be capable of such rearrangements. This appears to be the case; when purified [35 S]methionine/cysteine-labeled Sindbis virions are lysed in sample buffer and run on a nonreducing gel, five E1 species with mobilities similar to those of E1 β , - ζ , - δ , - γ , and - ϵ are resolved (Fig. 4B, lane V). It is unlikely that all of these species existed within the virions prior to lysis. Particularly, it is doubtful that five distinct E1 species within the Sindbis virus envelope could meet the requirements of the equivalent/quasi-equivalent interactions which are needed for virus assembly (8). Thus, the mature E1 and intracellular E1 which has progressed beyond the Golgi appear to be metastable species which are able to rearrange via thiol-disulfide exchange reactions initiated during or after virus or cell lysis.

Alkylating agents such as NEM and iodoacetamide (IAM) are commonly used to inhibit artifactual thiol-disulfide exchange reactions (10, 20, 34). However, the reaction of an alkylating agent with a free thiol is not instantaneous and can be restricted by the accessibility of a thiol within a protein or protein complex (10). Gidwitz et al. (19) have shown that mature Sindbis virions are unreactive with [14 C]IAM, indicating that any free thiols within the virus are not exposed. We have attempted unsuccessfully to prevent the formation of the multiple E1 species from lysed virions (Fig. 4B, lane V) by using a variety of methods, including incubating virus for up to 5 h in the presence of up to 0.1 M NEM or IAM prior to lysis. The use of nonionic or ionic detergents and lysis at low pH (5.3) and on ice (in order to slow thiol-disulfide exchange

FIG. 4. Comparison of the E1 species resolved from lysed virions and from cells lysed under different conditions. Purified SVHR (lane V) was lysed in $2\times$ sample buffer. SVHR-infected BHK cells were pulse-labeled for 5 min. The monolayers were then lysed in the absence of any alkylating agents, lysed in buffer containing 50 mM IAM, lysed in buffer containing 50 mM NEM, or incubated on ice with 20 mM NEM in PBS for 10 min before lysis in buffer containing 20 mM NEM (lanes 1 to 4, respectively). All samples were analyzed by SDS-PAGE under reducing (A) and nonreducing (B) conditions. A portion of the nonreducing stacking gel is included in the figure. The B protein indicated is seen in both reducing and nonreducing gels and has been shown to be an aberrant product consisting of uncleaved PE2-6K-E1 (38). This protein seems to be degraded during chases.

reactions) with alkylating agents present also did not block the formation of the multiple E1 species (results not shown).

Stability of nascent E1. Four E1 species, E1 α , - β , - δ , and - γ , can be detected prior to the production of E1 ϵ during pulse-chases (Fig. 1B and 3B). We have examined the influence of NEM treatment before and during cell lysis on the stability of the disulfide arrangements of these E1 species. Infected BHK cells were pulse-labeled for 5 min and then lysed under different conditions. E1 β and - γ , along with smaller amounts of E1 α and - δ , are recovered when cells are incubated with 20 mM NEM and then lysed in the presence of 20 mM NEM after the pulse (Fig. 4B, lane 4). Identical results are obtained when the cells are lysed without prior incubation with NEM in lysis buffer containing 50 mM NEM (Fig. 4B, lane 3). In contrast, when cells are lysed in the absence of treatment with an

FIG. 5. Model depicting the folding pathway of the E1 glycoprotein. See text for details. TGN, trans-Golgi network; PM, plasma membrane.

alkylating agent, the E1 species recovered resemble slightly faster migrating E1 α and - γ , a fainter E1 β , an enhanced E1 ζ , and E1 δ (Fig. 4B, lane 1). Additionally, when cell lysis is performed in the presence of 50 mM IAM without prior incubation with any alkylating agent, some of E1 α and all of E1 γ appear to migrate slightly faster in the gel, and E1 ζ and E1 δ levels are enhanced (Fig. 4B, lane 2). This pattern of E1 species is very similar to that recovered, regardless of treatment with either IAM or NEM, after virion lysis (Fig. 4B, lane V) or after lysis of cells which have been chased to allow most of E1 to progress beyond the Golgi (long chases in Fig. 1B and lanes 6, 7, and 11 in Fig. 3B). It thus appears that the early E1 species, like the mature (virion) and nearly mature (post-Golgi) E1 forms, are unstable with respect to their disulfide bonds. Artifactual disulfide formation and/or rearrangements within the early E1 forms during or after cell lysis, however, are more effectively inhibited by NEM treatment.

E1 folding pathway. In the absence of NEM treatment, the quantities of E1 ζ and - δ increase (Fig. 4B, lanes 1 and 2), suggesting that these two species are primarily products of artifactual thiol-disulfide exchange reactions. Therefore, E1 α , - β , and - γ appear to be the legitimate precursors to the E1 ϵ species. The probable precursor-product relationships among these E1 forms were determined by analysis of the nonreducing gel in Fig. 1B.

The integrated optical density of each E1 species resolved in this gel was measured and is presented in Fig. 1C as a percentage of the total E1 at each chase time after a 2-min pulse. By 5 min of chase, E1 α can no longer be detected, indicating that it is likely a short-lived precursor to the subsequent, faster-migrating E1 species. As E1 α decreases, the relative amount of E1 β increases and then declines. Concurrent with the decrease in E1 β is an increase in E1 γ , which then also begins to decrease. As E1 γ declines, E1 ϵ appears but is never recovered as the predominant E1 species. This is apparently due to the conversion of E1 ϵ , late in the secretory pathway, into a NEM-inaccessible, metastable conformation which rearranges during the isolation process (see Fig. 1C and 3B). These results suggest that E1 folding proceeds sequentially after translocation into the ER from E1 α to E1 β to E1 γ to E1 ϵ (see Fig. 5). The validity of this folding pathway is also supported by the results obtained from pulse-chases carried out with nocodazole- and BFA-treated cells (Fig. 3B, lanes 1 to 4). Although we cannot exclude the possibility that E1 δ is also a folding intermediate for E1 ϵ , the results already presented (Fig. 4B) make this unlikely.

The ability of mature E1 to rearrange, when triggered, via intramolecular thiol-disulfide exchange reactions into alternative species, which include its precursors E1 β and - γ , indicates that these species differ in the arrangement but not the number of their disulfide bonds. Thus, much of E1 folding, from E1 β to E1 γ to E1 ϵ , appears to occur via isomerization of existing disulfide bonds. That the thiol-disulfide exchange reactions which can be triggered within the various E1 species are intramolecular is implied by the inability to trap, in the

presence or absence of alkylating agents, significant amounts of any disulfide-linked multimeric intermediates which would necessarily form if intermolecular exchanges were involved (Fig. 4B and results not shown).

Participation of PE2/E2 in E1 folding. During the conduct of these experiments, we found that PE2/E2 did not demonstrate the disulfide rearrangements characteristic of E1 and that the electrophoretic mobility of PE2/E2 was similar on reducing and nonreducing gels (data not shown). Additionally, it was found that immunoprecipitations with E1-specific antisera consistently show more PE2 coprecipitating with E1 when the E1 ϵ form is present (see Fig. 1B and 3B; results not shown). These data suggest that pairing of PE2 with E1 may occur only after E1 has achieved its native, stable, export-competent conformation in the ER. Previous observations that temperature-sensitive defects in E1 block the export of PE2 from the ER imply that PE2 probably requires oligomerization with correctly folded E1 to become transport competent (16, 24).

DISCUSSION

A model, based on the above results, of the folding pathway of the Sindbis virus E1 glycoprotein is presented in Fig. 5. Upon translocation into the ER, E1 is present in an extended conformation, designated E1 α , which has an electrophoretic mobility similar to that of fully reduced E1 (Fig. 1). E1 α is rapidly converted into a faster-migrating, and presumably more compact (10), disulfide-stabilized species, E1 β . This species, in turn, is folded into a still more compact form, E1 γ . These three nascent E1 species apparently possess a free thiol(s) which can initiate artifactual intramolecular disulfide formation and/or rearrangements during or after cell lysis. This thiol(s), however, appears to be accessible to the alkylating agent NEM, which can inhibit, although not completely, these artifactual reactions (Fig. 4B). Prior to export from the ER, E1 γ is converted into the most compact of the E1 species recovered, E1 ϵ . This species, unlike its precursors, is stable with respect to its disulfide bonds regardless of treatment with NEM and remains so after processing by both ER and Golgi enzymes (Fig. 3B). However, probably at some point beyond the Golgi stacks, E1 ϵ undergoes a conformational change rendering it metastable with respect to its disulfide bonds and inaccessible to NEM (Fig. 1B and C and Fig. 3B). It is this compact, metastable, NEM-inaccessible E1 ϵ conformation which appears to be assembled into virions (Fig. 4B, lane V). Consequently, despite treatment with NEM, multiple disulfide-bonded E1 species are recovered from lysed virions (Fig. 4B, lane V) and from cells after E1 has been chased past the Golgi stacks (Fig. 1B) due to artifactual thiol-disulfide exchange reactions initiated within the metastable E1 ϵ species during its isolation.

The ability of the mature E1 ϵ species to rearrange (see Fig. 3B and 4B, lane V), when triggered appropriately, into multiple species which include its precursors E1 β and - γ suggests that much of the folding of E1 within the ER proceeds via internal isomerization of existing disulfide bonds. Folding into E1 ϵ may occur prior to the pairing of E1 and PE2 within the ER, as suggested by the observation that more PE2 coprecipitates with E1 when the E1 ϵ form is present (Fig. 1B and 3B and data not shown). Alternately, E1 may fold by using PE2 as a scaffold, as has been suggested to be the situation for the rubella virus envelope proteins (20). The conversion of the stable E1 ϵ species into a metastable, NEM-inaccessible conformation late in the secretory pathway corresponds roughly with the cleavage of PE2 into E2 and E3, an event thought to occur within the lower-pH environment of the trans-Golgi

network (11). This raises the possibility that these occurrences are intimately linked.

E1 is the most highly conserved of the alphavirus structural proteins (36), and its metastable nature may play a significant role in the life cycle of these viruses. E1-E1 associations stabilized by intramolecular disulfide bonds are responsible for maintaining the rigidly ordered protein lattice of the alphavirus envelope (2, 3, 32). During the infection process, these associations must be disrupted to allow membrane fusion and the subsequent release of the nucleocapsid into the cell's interior to initiate viral replication (1, 7). Workers in our laboratory have proposed that conformational changes induced within E1 by host cell-virus interaction (17, 30) may bring a thiol(s) into position to initiate specific intramolecular thiol-disulfide exchange reactions (1). The resulting disulfide rearrangements may then serve to destabilize critical E1 domains involved in maintaining the protein lattice of the envelope, allowing disassembly and membrane fusion. This scenario does not exclude the possibility that while certain E1-E1 interactions are dissolved, others are stabilized (42). Disulfide rearrangements within E1 could also trigger the expression of a fusion function thought to reside within this glycoprotein (25, 32, 42). Additionally, the differences in redox potentials and the changes in free energy as E1 rearranges from a high-energy, metastable native structure into alternative, more thermodynamically stable forms may drive other activities required to allow the viral and host cell membranes to approach each other and fuse.

ACKNOWLEDGMENTS

This research was supported by grants AI14710 and AI19545 from the U.S. Public Health Service and by funds appropriated by the state of Texas to the Cell Research Institute. Matthew Mulvey was supported by a predoctoral fellowship through NIH training grant 5T32 CA09583.

REFERENCES

- Abell, B. A., and D. T. Brown. 1993. Sindbis virus membrane fusion is mediated by reduction of glycoprotein disulfide bridges at the cell surface. *J. Virol.* **67**:5496-5501.
- Anthony, R. P., and D. T. Brown. 1991. Protein-protein interactions in the alphavirus membrane. *J. Virol.* **65**:1187-1194.
- Anthony, R. P., A. M. Paredes, and D. T. Brown. 1992. Disulfide bonds are essential for the stability of the Sindbis virus envelope. *Virology* **190**:330-336.
- Bonatti, S., G. Migliaccio, G. Blobel, and P. Walter. 1984. Role of signal recognition particle in membrane assembly of Sindbis viral glycoproteins. *Eur. J. Biochem.* **140**:499-502.
- Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83-88.
- Braakman, I., H. Hoover-Litty, K. R. Wagner, and A. Helenius. 1991. Folding of influenza hemagglutinin in the endoplasmic reticulum. *J. Cell Biol.* **114**:401-411.
- Brown, D. T., and L. D. Condreay. 1986. Replication of alphaviruses in mosquito cells, p. 171-207. *In* S. Schlesinger and M. J. Schlesinger (ed.), *The Togaviridae and Flaviviridae*. Plenum Press, New York.
- Brown, D. T., and J. Edwards. 1992. Structural changes in alphaviruses accompanying the process of membrane penetration. *Semin. Virol.* **3**:519-527.
- Caspar, D. L. D., and C. Klug. 1962. Physical principles in the construction of regular viruses. *Cold Spring Harbor Symp. Quant. Biol.* **27**:1-24.
- Copeland, L. S., K.-P. Zimmer, K. R. Wagner, G. A. Healey, I. Mellman, and A. Helenius. 1988. Folding, trimerization, and transport are sequential events in the biogenesis of influenza hemagglutinin. *Cell* **53**:197-209.
- Creighton, T. E. 1984. Disulfide bond formation in proteins. *Methods Enzymol.* **107**:305-329.
- De Curtis, I., and K. Simons. 1988. Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. *Proc. Natl. Acad. Sci. USA* **85**:8052-8056.
- de Silva, A., I. Braakman, and A. Helenius. 1993. Posttranslational folding of vesicular stomatitis virus G protein in the ER: involvement of noncovalent and covalent complexes. *J. Cell Biol.* **120**:647-655.
- Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius. 1993. Folding and assembly of viral membrane proteins. *Virology* **193**:545-562.
- Doms, R. W., G. Russ, and J. W. Yewdell. 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* **109**:61-72.
- Earl, P. L., B. Moss, and R. W. Doms. 1991. Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. *J. Virol.* **65**:2047-2055.
- Erwin, C., and D. T. Brown. 1980. Intracellular distribution of Sindbis virus membrane proteins in BHK-21 cells infected with wild-type virus and maturation-defective mutants. *J. Virol.* **36**:775-786.
- Flynn, D. C., W. J. Meyer, J. M. Mackenzie, Jr., and R. E. Johnston. 1990. A conformational change in Sindbis virus glycoproteins E1 and E2 is detected at the plasma membrane as a consequence of early virus-cell interaction. *J. Virol.* **64**:3643-3653.
- Freedman, R. B. 1992. Protein folding in the cell, p. 455-537. *In* T. E. Creighton (ed.), *Protein folding*. W. H. Freeman and Company, New York.
- Gidwitz, S., J. M. Polo, N. L. Davis, and R. E. Johnston. 1988. Differences in virion stability among Sindbis virus pathogenesis mutants. *Virus Res.* **10**:225-240.
- Hobman, T. C., L. Woodward, and M. G. Farquhar. 1993. The rubella virus E2 and E1 spike glycoproteins are targeted to the Golgi complex. *J. Cell Biol.* **121**:269-281.
- Hurtley, S., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **5**:277-307.
- Kim, P. S., D. Bole, and P. Arvan. 1992. Transient aggregation of nascent thyroglobulin in the endoplasmic reticulum: relationship to the molecular chaperon, BiP. *J. Cell Biol.* **118**:541-549.
- Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116**:1071-1080.
- Knipfer, M. E., and D. T. Brown. 1989. Intracellular transport and processing of Sindbis virus glycoproteins. *Virology* **170**:117-122.
- Levy-Mintz, P., and M. Kielian. 1991. Mutagenesis of the putative fusion domain of the Semliki Forest virus spike protein. *J. Virol.* **65**:4292-4300.
- Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H.-P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell* **60**:821-836.
- Liu, N., and D. T. Brown. 1993. Transient translocation of the cytoplasmic (endo) domain of a type I membrane glycoprotein into cellular membranes. *J. Cell Biol.* **120**:877-883.
- Liu, N., and D. T. Brown. 1993. Phosphorylation and dephosphorylation events play critical roles in Sindbis virus maturation. *Virology* **196**:703-711.
- Metsikkö, K., and H. Garoff. 1990. Oligomers of cytoplasmic domain of the p62/E2 membrane protein of Semliki Forest virus bind to nucleocapsid in vitro. *J. Virol.* **64**:4678-4683.
- Meyer, W. J., and R. E. Johnston. 1993. Structural rearrangement of infecting Sindbis virions at the cell surface: mapping of newly accessible epitopes. *J. Virol.* **67**:5114-5125.
- Olson, T. S., M. J. Bamberger, and M. D. Lane. 1988. Post-translational changes in tertiary and quaternary structure of the insulin receptor. *J. Biol. Chem.* **263**:7342-7351.
- Omar, A., and H. Koblet. 1988. Semliki Forest virus particles containing only the E1 envelope glycoprotein are infectious and can induce cell-cell fusion. *Virology* **166**:17-23.
- Paredes, A. M., D. T. Brown, R. Rothnagel, W. Chiu, R. J. Schoepp, R. E. Johnston, and B. V. V. Prasad. 1993. Three-dimensional structure of a membrane-containing virus. *Proc. Natl.*

- Acad. Sci. USA **90**:9095–9099.
34. **Persson, R., and R. F. Pettersson.** 1991. Formation and intracellular transport of a heterodimeric viral spike protein complex. *J. Cell Biol.* **112**:257–266.
 35. **Renz, D., and D. T. Brown.** 1976. Characteristics of Sindbis virus temperature-sensitive mutants in cultured BHK-21 and *Aedes albopictus* (mosquito) cells. *J. Virol.* **19**:775–781.
 36. **Rice, C. M., and J. H. Strauss.** 1982. Association of Sindbis virion glycoproteins and their precursors. *J. Mol. Biol.* **154**:325–348.
 37. **Scheefers, H., U. Scheefers-Borchel, J. Edwards, and D. T. Brown.** 1980. Distribution of virus structural proteins and protein-protein interactions in plasma membrane of baby hamster kidney cells infected with Sindbis or vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* **77**:7277–7281.
 38. **Schlesinger, M. J., and L. Kääriäinen.** 1980. Translation and processing of alphavirus proteins, p. 371–392. *In* R. W. Schlesinger (ed.), *The togaviruses*. Academic Press, New York.
 39. **Schlesinger, M. J., and S. Schlesinger.** 1986. Formation and assembly of alphavirus glycoproteins, p. 121–148. *In* S. Schlesinger and M. J. Schlesinger (ed.), *The Togaviridae and Flaviviridae*. Plenum Press, New York.
 40. **Strauss, E., and J. H. Strauss.** 1986. Structure and replication of the alphavirus genome, p. 35–82. *In* S. Schlesinger and M. J. Schlesinger (ed.), *The Togaviridae and Flaviviridae*. Plenum Press, New York.
 41. **Vidal, S., G. Mottot, D. Kolakofsky, and L. Roux.** 1989. Addition of high-mannose sugars must precede disulfide bond formation for proper folding of Sendai virus glycoproteins. *J. Virol.* **63**:892–900.
 42. **Wahlberg, J. M., and H. Garoff.** 1992. Membrane fusion process of Semliki Forest virus. I. Low-pH-induced rearrangement in spike protein quaternary structure precedes virus penetration into the cells. *J. Cell. Biol.* **116**:339–348.
 43. **Wust, C. J., J. A. W. Nicholas, D. Fredin, D. C. Dodd, R. J. Brideau, M. E. Levely, and A. Brown.** 1989. Monoclonal antibodies that cross-react with the E1 glycoprotein of different alphavirus serogroups: characterization including passive protection *in vivo*. *Virus Res.* **13**:101–112.
 44. **Ziemiecki, A., H. Garoff, and K. Simons.** 1980. Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. *J. Gen. Virol.* **50**:111–123.