JOURNAL OF VIROLOGY, Mar. 1995, p. 1621–1627
0022-538X/95/$04.00+0

Vol. 69, No. 3

Involvement of the Molecular Chaperone BiP in Maturation of Sindbis Virus Envelope Glycoproteins

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Received 4 October 1994/Accepted 30 November 1994

Sindbis virus codes for two membrane glycoproteins, E1 and PE2, which assemble into heterodimers within the endoplasmic reticulum. We have examined the role of the molecular chaperone BiP (grp78) in the maturation of these two proteins. E1, which folds into its mature conformation via at least three intermediates differing in the configurations of their disulfide bonds, was found to interact strongly and transiently with BiP after synthesis. ATP depletion mediated by carbonyl cyanide *m*-chlorophenylhydrazone treatment results in the stabilization of complexes between BiP and E1. The depletion of intracellular ATP levels also greatly inhibits conversions between the E1 folding intermediates and results in the slow incorporation of E1 into disulfide-stabilized aggregates. These results suggest that the ATP-regulated binding and release of BiP have a role in modulating disulfide bond formation during E1 folding. In comparison with E1, very little PE2 is normally recovered in association with BiP. However, under conditions in which E1 folding is aberrant, increased amounts of PE2 become directly associated with BiP. The formation of these BiP-PE2 interactions occurs after E1 begins to misfold or fails to fold efficiently. We propose that nascent PE2 is stable prior to pairing with E1 for only a limited period of time, after which unpaired PE2 becomes recognized by BiP. This implies that the productive association of PE2 and E1 must occur within a restricted time frame and only after E1 has accomplished certain folding steps mediated by BiP binding and release. Kinetic studies which show that the pairing of E1 with PE2 is delayed after translocation support this conclusion.

Sindbis virus is the prototype alphavirus, and its structure, in comparison with that of other enveloped viruses, has been well characterized. The Sindbis virion possesses a nucleocapsid core consisting of a 42S, single-stranded, positive-polarity RNA genome in association with 240 copies of a basic capsid protein (C). A host-derived membrane bilayer containing the viral glycoproteins E1 and E2 envelopes the nucleocapsid. Eighty tightly complexed trimers of E1 and E2 heterodimers are organized into a rigid T=4 icosohedral lattice that mirrors the symmetry of the underlying nucleocapsid core (40). The structural proteins of Sindbis virus are synthesized from a subgenomic 26S RNA in the order NH_2-C-PE2-6K-E1-COOH (43). Capsid protein is autoproteolytically cleaved from the developing polyprotein, becoming available for assembly into a nucleocapsid particle within the cytosol. The remaining polyprotein, directed by a signal sequence in the newly exposed N-terminal end of PE2, is cotranslationally translocated into the rough endoplasmic reticulum (ER). This polyprotein is cleaved by signal peptidase within the ER and modified by N-glycosylation and fatty acid acylation (43, 45). PE2 (the precursor of E2) and E1, which are both type I integral membrane glycoproteins, form a noncovalently associated hetero-oligomeric complex within the ER and are subsequently transported to the cell surface via the Golgi apparatus (42, 49). Late in this pathway, the majority of PE2 is processed into E2 and E3, a small glycopeptide that is released into the medium (43). The cytoplasmic tail of E2 interacts specifically with capsid protein, and such interactions lead to envelopment of the nucleocapsid during virus budding at the plasma membrane and help organize the strict geometry of the viral envelope proteins (29, 32–34).

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The structural integrity of the rigid protein lattice within the Sindbis virus envelope appears to be dependent upon intramolecular disulfide bonds residing within the E1 glycoprotein (2, 3). It has been proposed that these disulfide bonds must be rearranged during the initial steps of the infection process to allow disassembly of the virion (1). Therefore, the arrangement of disulfide bonds within E1 is critical during both virus assembly and disassembly. Both E1 and PE2 contain 16 highly conserved cysteine residues within their ectodomains (10, 14, 20, 26, 30, 47). The formation of disulfide bonds during protein folding is often accompanied by a change in the mobility of a protein in nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12). We have previously described the folding of E1 and PE2 by examining the changes in the electrophoretic mobilities, in nonreducing gels, of these proteins as they mature (37). E1 was found to fold into its mature conformation within the ER via at least three folding intermediates, differing in the configurations of their disulfide bonds. Posttranslational isomerization of disulfide bonds within E1 appears to occur during these folding events. Additionally, we have shown that E1 becomes metastable with respect to the arrangement of its disulfide bonds late in the secretory pathway prior to its assembly into virus. In contrast with E1, no PE2 folding intermediates were detected (37).

The folding and oligomerization of nascent proteins entering the lumen of the rough ER, rather than being completely spontaneous events, are often modulated by ER resident folding enzymes and chaperone proteins (23). Among the chaperones present within the ER is BiP (grp78), a member of the 70-kDa heat shock protein family (11, 23). BiP is a major component of the ER and has been found to associate transiently with a variety of folding and assembly intermediates and to interact more permanently with misfolded, aggregated, or unassembled proteins that fail to be transported from the ER (reviewed in references 11, 22, and 24). BiP also appears to

have a role in the translocation of proteins across the ER membrane (8). The C-terminal domain of BiP is thought to recognize extended, hydrophobic segments of polypeptide chain which are probably normally located within the interior of completely folded and assembled protein (5, 19). It is believed that the binding of BiP to its target polypeptides prevents the formation of inappropriate intra- and intermolecular interactions during protein folding (23). The binding and release of BiP are regulated by a weak ATPase activity present within the N terminus of the chaperone (38, 39).

In this study, we have investigated the involvement of BiP in the maturation of the Sindbis virus envelope glycoproteins. We provide data suggesting that interactions with BiP help modulate the formation of correct disulfide bonds within nascent E1. Additionally, results are presented which show that aberrant E1 folding can lead to the direct association of PE2 with BiP.

MATERIALS AND METHODS

Cells and viruses. BHK-21 cells were cultured at 37°C in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (GIBCO), 5% tryptose phosphate broth, and 2 mM L-glutamine. Heat-resistant Sindbis virus (SVHR) and the temperature-sensitive mutant TS23 were both originally provided by E. R. Pfefferkorn (Dartmouth Medical College). Virus stocks were prepared as described previously (41).

Infection, drugs, and radiolabeling. As described previously (37), subconfluent BHK monolayers were treated with medium containing dactinomycin (4 μg/ml; Calbiochem) for 1 h at 37°C prior to infection with ~100 PFU of either SVHR or TS23 per cell. At 4 h postinfection, cells were starved briefly (10 to 15 min) in methionine- and cysteine-free medium and then pulse-labeled with [^{35}S]methionine/cysteine protein labeling mix (New England Nuclear) at a concentration of 50 μCi/ml. All chases were performed with complete medium containing excess nonradioactive methionine and 75 μg of cycloheximide per ml. Incubations of TS23-infected cells were done at the nonpermissive temperature (39.5°C). SVHR-infected cells were incubated at 37°C. Dactinomycin treatment and a prelabeling starvation period were omitted for the experiment illustrated in Fig. 2. Where indicated, cells were treated with 5 μg of brefeldin A (BFA; Epicentre Technologies) per ml for 1 h prior to the pulse and throughout the pulse-chases. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Aldrich) was prepared in dimethyl sulfoxide and used, where indicated, in the chase medium at a final concentration of 300 μg/ml.

Immunoprecipitations and SDS-PAGE. Following the pulse-chases, monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) containing cycloheximide. Where indicated, cells were then incubated on ice with 20 mM *N*-ethylmaleimide (NEM) in PBS for ~10 min and then lysed in buffer containing NEM. For the experiment shown in Fig. 3, cell lysates were prepared and immunoprecipitated with affinity-purified anti-E1 polyclonal antibodies conjugated to Sepharose beads (Sigma) as previously described (37). These samples were analyzed by reducing and nonreducing SDS-PAGE on 12.5% polyacrylamide gels. For all other experiments, cells were lysed in buffer containing 1% Triton X-100, 150 mM NaCl, and 10 mM Tris (pH 7.2) plus 0.2 mM each of the protease inhibitors phenylmethylsulfonyl fluoride, *N*-tosyl-L-phenylalanine chloromethyl ketone, and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (Sigma). Nuclei and large cell debris were pelleted and discarded. Lysates were then reacted with 5 μg of anti-BiP monoclonal antibody (StressGen Biotechnologies Corp.), with anti-vesicular stomatitis virus (VSV) G protein antiserum, or with monoclonal antibodies specific for either E1 or E2 for 2 h at 4°C. Anti-E1 antibody (SIN 33) and anti-E2 antibody (R15) were kindly provided by A. L. Schmaljohn (U.S. Army Medical Research Institute of Infectious Diseases) and R. E. Johnston (University of North Carolina), respectively (35, 44). Immune complexes were recovered by gently rocking lysates for 1 h at 4°C with 100 μl of a 10% solution of protein A insolubilized on Sepharose CL-4B beads (Sigma) in lysis buffer. The bead-protein complexes were washed three times with buffer containing 0.1% Triton X-100, 150 mM NaCl, 10 mM Tris, and 0.2 mM phenylmethylsulfonyl fluoride (pH 7.2), resuspended in 30 μl of 2× reducing sample buffer, and heated for 4 min at 100°C. Supernatants (equal volumes) were analyzed by SDS-PAGE on 10.8% polyacrylamide gels as previously described (3). Fluorography was performed by the method of Bonner and Laskey (6). The nonreducing gel shown in Fig. 3B was enhanced as previously described (37) to increase contrast between bands.

RESULTS

E1 transiently associates with BiP. To determine if BiP associates with Sindbis virus proteins, SVHR-infected BHK monolayers were pulse-labeled for 5 min, chased for various times up to 20 min, lysed, and immunoprecipitated with anti-BiP monoclonal antibody. Three Sindbis proteins, identified as B protein (p110), PE2, and E1, coprecipitate with BiP immediately after the pulse (Fig. 1, lane 1). The recovery of these proteins with anti-BiP antibody is specific, as determined by two criteria. First, no Sindbis proteins are recovered after a pulse from lysates with control anti-VSV G protein antibody (lane 7). Second, if cell lysis is performed in the presence of excess ATP, the levels of B protein, PE2, and E1 coprecipitated with BiP are dramatically reduced (lane 6). This latter result is in agreement with previous data showing that the addition of ATP to cell extracts causes the disruption of BiP-substrate complexes (38).

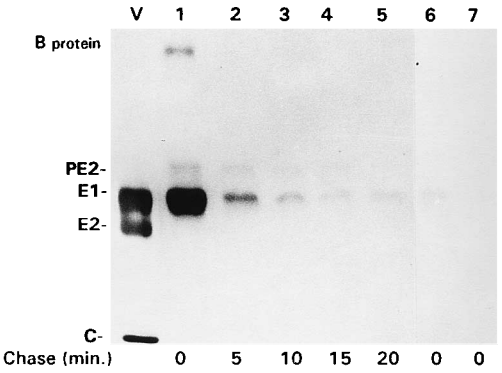

FIG. 1. Interaction of BiP with Sindbis virus proteins. BHK cells, at 4 h postinfection with SVHR, were pulse-labeled for 5 min with [^{35}S]methionine/cysteine and then chased for the times indicated. Cells were lysed normally or in buffer containing 1 mM excess ATP (lane 6). Lysates were immunoprecipitated with anti-BiP monoclonal antibody (lanes 1 to 6) or with anti-VSV G protein antibody (lane 7), and immunoprecipitates were analyzed by reducing SDS-PAGE. Lane V, Sindbis virus marker proteins.

It has been shown that Sindbis virus infection can induce the synthesis of certain heat shock proteins (21), raising the possibility that increased production of BiP may also occur in Sindbis virus-infected cells. The constitutive synthesis of BiP, however, along with the synthesis of most other host proteins, is inhibited in Sindbis virus-infected BHK cells with or without dactinomycin treatment (Fig. 2) (48). Therefore, radiolabeled BiP is not detected in the pulse-chase experiments used for Fig. 1.

B protein, which consists of uncleaved PE2, 6K, and E1, has been characterized as a nonglycosylated by-product of Sindbis virus infection (18, 45). Its specific and transient coimmunoprecipitation with BiP indicates that some B protein can be at least partially integrated into the lumen of the ER (Fig. 1, lane 1). This BiP-associated B protein appears to be partially gly-

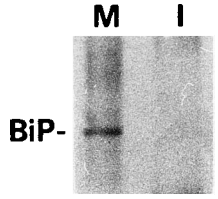

FIG. 2. BiP synthesis is inhibited in SVHR-infected cells. BHK cells, untreated with dactinomycin, were either mock infected (lane M) or infected with SVHR (lane I). After a 4-h incubation at 37°C, cells were pulse-labeled with [^{35}S]methionine/cysteine for 30 min and then lysed. Lysates were immunoprecipitated with anti-BiP monoclonal antibody. Radiolabeled BiP is only recovered from the mock-infected cells.

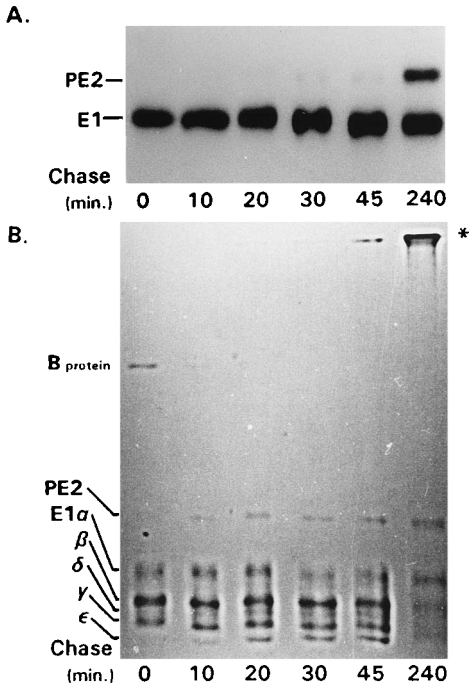

FIG. 3. Effect of CCCP treatment on E1 folding. SVHR-infected BHK cells were pulse-labeled for 2 min and then chased in medium containing CCCP (300 μg/ml) for the times indicated. Cells were then treated with NEM on ice prior to and during lysis. E1 was immunoprecipitated from the lysates and analyzed by electrophoresis through 12.5% polyacrylamide gels under reducing (A) and nonreducing (B) conditions (37). Disulfide-stabilized aggregates at the interface between the nonreducing stacking and resolving gels are indicated (*).

cosylated (data not shown) and may represent the product of an aborted translocation attempt.

Among the Sindbis proteins which associate with BiP, E1 is clearly the most prominent (Fig. 1, lane 1). Equal amounts of E1 and PE2 are synthesized in infected cells, yet vastly more E1 than PE2 coprecipitates with BiP following the pulse. The level of E1 associated with BiP decreases rapidly during chases (lanes 2 to 5). These data suggest that the transient interaction of BiP with E1 has a role in normal E1 maturation.

ATP depletion inhibits E1 folding. ATP binding stimulates the disassociation of BiP from substrate proteins (39), and the ATP-dependent folding of at least several proteins within the ER is thought to reflect the involvement of BiP in the folding process (7, 16). The association of nascent E1 with BiP (Fig. 1) indicates that E1 folding might also be dependent upon ATP. To determine whether this is the case, infected BHK cells were pulse-labeled for 2 min and then chased for various times in medium containing CCCP, an uncoupler of oxidative phosphorylation (24). CCCP treatment depletes intracellular ATP levels and, at the concentration used in this study, blocks the export of Sindbis proteins from the ER to the Golgi stacks (32). Following the pulse-chases, cells were incubated on ice with NEM and then lysed in the presence of NEM as described in Materials and Methods in order to inhibit artifactual disulfide bond formation during or after cell lysis (12, 37). Cell lysates were then immunoprecipitated with anti-E1 antiserum, and samples were examined by SDS-PAGE under reducing (Fig. 3A) and nonreducing (Fig. 3B) conditions. In the reducing gel, E1 migrates as a single band after all chase times (Fig. 3A). In the nonreducing gel, E1 folding intermediates that differ in the number and/or arrangement of their disulfide bonds are resolved (Fig. 3B). These E1 species appear to be identical to those recovered without CCCP treatment (37), but their behavior during chases differs markedly.

Normally, the folding of E1 within the ER proceeds sequentially through three folding intermediates, designated E1α, -β, and -γ, into a compact disulfide-bonded conformation, E1ε (37). Two other E1 species, E1δ and -ζ, have also been identified, but their role, if any, in E1 maturation is unclear. The results shown in Fig. 3B indicate that ATP depletion significantly retards E1 folding. Specifically, the E1α species, which normally folds completely into E1β within 5 min after a 2-min pulse (37), persists as a prominent band for up to 4 h of chase in the presence of CCCP (Fig. 3B). The folding steps from E1β to E1γ to E1ε also appear to be greatly inhibited in ATP-depleted cells. We have shown previously that when protein transport from the ER is arrested by treatment of cells with the drug BFA, which has little effect on intracellular ATP levels (36), the vast majority of E1 is folded into the stable E1ε species within 2 h of chase (37). In contrast, when the transport of proteins from the ER is blocked by ATP depletion, very little E1 reaches the E1ε conformation even after 4 h of chase (Fig. 3B).

In addition to the inhibition of E1 folding, chases in the presence of CCCP also result in the slow formation of large, disulfide-stabilized aggregates, which are detected at the top of the resolving gel shown in Fig. 3B. Reduction of these aggregates reveals that they are composed of both E1 and PE2 (Fig. 3A and data not shown). Aggregation of nascent viral proteins upon energy depletion has been described previously for VSV G protein (15) and influenza virus hemagglutinin (7). The incorporation of these viral glycoproteins into disulfide-stabilized aggregates upon ATP depletion occurs very rapidly (<10 min) in comparison to the 4 h required for large amounts of E1 to enter similar aggregates in energy-depleted cells (Fig. 3B). As pointed out by others (9), ATP is not required to maintain the oxidizing conditions within the ER needed for disulfide bond formation. Therefore, the inefficient folding and eventual aggregation of E1 shown in Fig. 3 are best explained by the inability of BiP to function properly in ATP-depleted cells. However, we cannot rule out other effects that ATP depletion mediated by CCCP treatment may have on E1 folding.

E1 remains associated with BiP in CCCP-treated cells. Dorner et al. (17) have shown that transient complexes between BiP and maturing factor VIII protein within the ER can be stabilized with CCCP treatment. This observation provided evidence that the ATP-dependent release of BiP from its target polypeptides seen in earlier in vitro experiments is also relevant in vivo. To investigate whether transient BiP-E1 complexes are also stabilized with CCCP treatment, infected BHK cells were pulse-labeled for 5 min and then chased in the presence of CCCP for up to 45 min. Cell lysates were prepared and immunoprecipitated with anti-BiP monoclonal antibody as in Fig. 1. The results, shown in Fig. 4A (lanes 1 to 4; compare with lanes 1 to 5 in Fig. 1), indicate that ATP depletion mediated by CCCP treatment does prevent the disassociation of BiP-E1 complexes. These data, in conjunction with the results presented in Fig. 1 and 3, support a role for the ATP-regulated binding and release of BiP in the normal folding pathway of E1.

Formation of BiP-PE2 complexes. PE2 is usually recovered in only small amounts from lysates by using anti-BiP antibody (Fig. 1). The results presented in Fig. 4A (lanes 1 to 4), however, show that significantly increased levels of PE2 coprecipitate with BiP during chases in CCCP-treated cells. Following a 20-min chase with CCCP treatment, the amounts of E1 and PE2 coprecipitated from lysates with BiP are similar (lane 3). The explanation that PE2 coprecipitates with BiP in this situ-

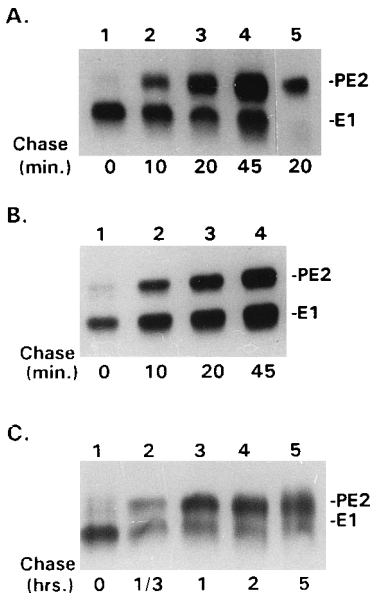

FIG. 4. Induction of prolonged and/or increased associations of E1 and PE2 with BiP. The coprecipitation of E1 and PE2 with BiP was examined in three separate situations in which the fidelity of E1 folding is compromised. (A) SVHR-infected BHK cells were pulse-labeled for 5 min and then chased for the times indicated in medium containing 300 μg of CCCP per ml. (B) TS23-infected BHK cells, incubated at the nonpermissive temperature (39.5°C), were pulse-labeled for 5 min and chased for the indicated times. (C) BHK cells, 3 h postinfection with SVHR, were treated for 1 h with medium containing 5 μg of BFA per ml. Cells were then pulse-labeled for 5 min and chased for the indicated times in the presence of BFA. Following the pulse-chases (A through C), cell lysates were prepared and immunoprecipitated with anti-BiP monoclonal antibody. Alternately, cell lysates were first immunoprecipitated several times with anti-E1 antiserum before a final immunoprecipitation with anti-BiP antibody was performed (panel A, lane 5). Samples were analyzed by reducing SDS-PAGE.

ation solely because of PE2 interactions with E1 molecules which are associated with BiP is not valid. E1 can be depleted from cell lysates by serial immunoprecipitations with anti-E1 antiserum, and PE2 but not E1 will still be recovered in large amounts with anti-BiP antibody (Fig. 4A, lane 5; compare with lane 3). This result indicates that PE2, in CCCP-treated cells, can become directly complexed with BiP. This observation suggested that perhaps ATP is required to prevent PE2 from misfolding into a conformation which is recognized by BiP. More specifically, rather than a direct requirement for ATP in PE2 maturation, it is possible that the inefficient or incorrect folding of E1 in ATP-depleted cells is responsible for the formation of BiP-PE2 complexes. The use of the temperature-sensitive Sindbis virus mutant TS23 provided an opportunity to test this possibility.

TS23 was derived from SVHR by Burge and Pfefferkorn (9) and has two amino acid substitutions at positions 106 and 267 in the E1 glycoprotein (4). At the nonpermissive temperature (39.5°C) in TS23-infected cells, E1 fails to fold correctly, and within a 2-min pulse, a substantial portion of nascent E1 is incorporated into disulfide-stabilized aggregates (unpublished observation). Additionally, the transport of both E1 and PE2 from the ER is inhibited (27). The PE2 sequence of TS23 is the same as in SVHR (4). Thus, the misfolding of mutant E1 protein influences the maturation of wild-type PE2. The effect of inefficient E1 folding, induced without ATP depletion, on the association of PE2 with BiP was investigated by using TS23 along with pulse-chase and coimmunoprecipitation protocols as already described. Immediately after a 5-min pulse of TS23-infected cells at the nonpermissive temperature, E1 and very little PE2 are coprecipitated with anti-BiP monoclonal antibody (Fig. 4B, lane 1). This result is similar to that seen after a 5-min pulse of SVHR-infected cells (Fig. 1, lane 1). Thus, E1 misfolding does not immediately result in an increase in the amount of PE2 complexed with BiP. However, with chases, greatly increased levels of PE2 coprecipitate with BiP (Fig. 4B, lanes 2 to 4), as also seen in SVHR-infected CCCP-treated cells (Fig. 4A). As might be expected, large amounts of misfolded mutant TS23 E1 protein associate with BiP throughout chases at the nonpermissive temperature (Fig. 4B). E1 and PE2 synthesized in wild-type virus-infected cells show no differences in their interactions with BiP at 39.5 versus 37°C (data not shown).

These data indicate that PE2 does not necessarily become complexed with BiP in CCCP-treated cells because of a direct requirement for ATP in the maturation of PE2. Instead, it appears that PE2 associates with BiP in a delayed fashion as a consequence of the failure of E1 to fold efficiently. The results shown in Fig. 4A and B suggest that nascent PE2 is stable prior to pairing with E1 for only a limited period of time, after which unpaired PE2 becomes recognized by BiP. This implies that the pairing of PE2 and E1 within the ER must occur within a limited time frame, and only after E1 has completed folding into an appropriate conformation.

Interaction of BiP with PE2 and E1 in BFA-treated cells. Sindbis virus glycoproteins normally exit the ER with a half-time of approximately 17 to 20 min (27, 37). Since proteins must typically fold and oligomerize correctly before exiting the ER (16, 25), it is assumed that E1 folds and pairs with PE2 with a half-time of less than 20 min. However, in cells treated with BFA (which induces the redistribution of the Golgi stacks into the ER and inhibits anterograde transport from the ER [31, 36]), 1 to 2 h are required for most of E1 to fold into the mature E1ε conformation (37 and data not shown). Thus, a population of E1 molecules in BFA-treated cells appears to fold with slower than normal kinetics into the E1ε species. Furthermore, a small fraction of E1 proteins in BFA-treated cells become incorporated into disulfide-stabilized aggregates during chases (data not shown). The use of BFA therefore provides an additional opportunity to test if the efficiency of E1 folding effects whether PE2 does or does not become complexed with BiP.

BHK cells were treated with BFA for 1 h at 3 h postinfection with SVHR. The cells were then pulse-labeled for 5 min and chased for various times (up to 5 h) as described in Materials and Methods with medium containing BFA. Cell lysates were then prepared and immunoprecipitated with anti-BiP monoclonal antibody. Just after the pulse, as seen without drug treatment (Fig. 1, lane 1), E1 and a very small amount of PE2 are coprecipitated with BiP (Fig. 4C, lane 1). BFA treatment therefore does not influence the level of newly synthesized PE2 which is recovered with BiP immediately after the pulse. With chases, however, as in CCCP-treated cells (Fig. 4A) and in cells infected with TS23 at the nonpermissive temperature (Fig. 4B), increased amounts of PE2 begin to associate with BiP (Fig. 4C, lanes 2 to 5). These observations support the previous proposition that the efficiency of E1 folding can effect whether BiP-PE2 complexes are formed in greater than normal amounts.

The level of E1 associated with BiP in BFA-treated cells decreases after the pulse and then remains fairly steady for up to 5 h of chase (Fig. 4C). The decrease in the amount of E1 coprecipitated with BiP between 0 and 20 min of chase (lanes 1 and 2) is likely due to a population of E1 molecules which completely fold and pair with PE2 within this time frame and

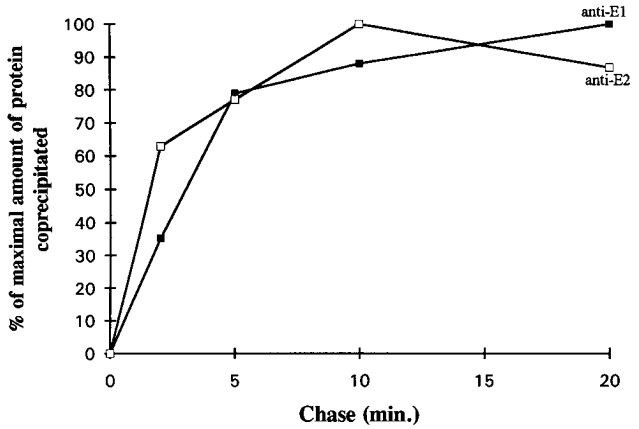

FIG. 5. Kinetics of E1 association with PE2. SVHR-infected cells were pulse-labeled for 2 min and then chased for the indicated times. Cells were then treated with NEM and lysed as described in Materials and Methods. Duplicate lysates were immunoprecipitated with anti-E1 monoclonal antibody (SIN 33) or with anti-E2 monoclonal antibody (R15). Immunoprecipitates were subjected to reducing SDS-PAGE. At each chase time, the percentage of the maximal amount of E1 which coprecipitates with PE2/E2 during chases was determined by scanning densitometry (□). The amount of PE2/E2 coprecipitating with E1 at each chase time was calculated similarly (■).

thus are no longer recognized by BiP. A decrease in the number and/or affinity of BiP binding sites on maturing E1 proteins could also contribute to a reduction in the amount of E1 recovered with BiP during chases. The E1 remaining associated with BiP after 5 h of chase in BFA-treated cells appears to be present in disulfide-stabilized aggregates (data not shown). We have also found that the PE2 which does not pair with E1 during chases in BFA-treated cells becomes irreversibly incorporated into disulfide-stabilized aggregates, containing little if any E1, and it is the PE2 within these aggregates that interacts with BiP (unpublished observations).

It is unclear why some E1 misfolds or folds slowly in BFA-treated cells. However, it is possible that the increased amounts of both viral and host proteins which enter and remain within the ER in the presence of BFA, coupled with the inhibition of BiP synthesis in SVHR-infected cells (Fig. 2), could result in a decrease in the local concentration of BiP within the ER. In this situation, only a limited number of nascent E1 proteins may have access to BiP at any one time. These E1 molecules, facilitated by BiP binding and release, could complete proper folding within a normal time frame, leaving the remaining E1 proteins without chaperones and unable to fold as efficiently. Other unknown factors affected by BFA treatment may also influence the folding and pairing of E1 and PE2 within the ER.

Kinetics of E1 and PE2 association. The preceding results suggest that E1 must complete certain folding steps after synthesis before reaching a conformation in which it can productively pair with PE2. This implies that the pairing of E1 with PE2 is not immediate after translocation into the ER. To determine a time course for the association of E1 with PE2, SVHR-infected BHK cells were pulse-labeled for 2 min and then chased for various times up to 20 min. Cells were then treated with NEM prior to and during lysis as described in Materials and Methods. Duplicate lysates were immunoprecipitated with either anti-E1 or anti-E2 monoclonal antibodies. The amount of E1 coprecipitating with PE2/E2 and the amount of PE2/E2 coprecipitating with E1 were determined for each chase time. The results, shown in Fig. 5, indicate that the time required for half-maximal association between E1 and PE2 is between 1 and 4 min. E1 and PE2/E2 coprecipitate with one another in increasing amounts during chases as more E1 folds into the E1ε species, which is readily detected by 10 min of chase after a 2-min pulse (37). These data agree with our previous observation that immunoprecipitations with anti-E1 polyclonal antiserum show more PE2 coprecipitating with E1 when the E1ε form is present.

DISCUSSION

We have previously shown that E1 folds sequentially within the ER through three increasingly compact folding intermediates, designated E1α, -β, and -γ, into a final disulfide-stabilized species, E1ε (37). These species differ in the arrangement and/or number of their disulfide bonds. E1α, the first intermediate in the proposed E1 folding pathway, has an electrophoretic mobility similar to that of fully reduced E1. This suggests that E1α possesses few, if any, of the disulfides present in the later E1 species and that most of the disulfide bonds within E1 form posttranslationally. Late in the secretory pathway, E1ε becomes metastable with respect to the arrangement of its disulfide bonds. During isolation from cells or virus, the metastable E1ε species rearranges, apparently through intramolecular thiol-disulfide exchange reactions, into alternate species. Two of these have mobilities which are identical to the E1ε precursors E1β and -γ. This indicates that the conversions between some of the E1 species during folding within the ER probably involve isomerization of intermediate, nonnative disulfide bonds. Further characterization of the disulfides within the E1 folding intermediates is needed to test this possibility.

Whether, and which, cysteine residues form disulfide bonds is largely determined by the conformation(s) acquired by a protein during folding (12). Once formed, disulfides can confer substantial stability on the conformation of a protein (13). The ability of the molecular chaperone BiP to reversibly bind to regions of polypeptide and prevent them from interacting with other regions gives BiP the potential to guide the formation or reshuffling of disulfide bonds during protein folding within the ER. We have found that BiP associates strongly and transiently with newly synthesized E1 under normal circumstances (Fig. 1). Upon ATP depletion mediated by CCCP treatment, E1 and BiP become more permanently complexed (Fig. 4A). Additionally, each folding step in E1 maturation, from E1α to E1β to E1γ to E1ε, appears to be sensitive to ATP depletion (Fig. 3). These results suggest that the ATP-regulated binding and release of BiP have a crucial role in modulating the formation, and possible rearrangements, of disulfide bonds during E1 folding.

Segal et al. (46) have proposed that the binding of BiP to nascent influenza virus hemagglutinin shields certain cysteine residues during translocation and folding, preventing the formation of nonnative disulfide bonds. In the case of the Sindbis virus E1 protein, our data suggest that interactions with BiP may help guide the posttranslational formation of an initial set of disulfide bonds (some of which are probably nonnative) during the conversion from E1α to E1β. Subsequent folding steps, which appear to be ATP dependent and which likely involve disulfide bond rearrangements, to form the E1ε species may also be mediated by BiP binding and release. The continued expression of BiP binding sites on the surface of misfolded and/or inefficiently folding E1 molecules results in prolonged associations between BiP and E1 (Fig. 4). These associations are likely responsible for inhibiting the export of misfolded and unfolded E1 from the ER. The synthesis of BiP, which has a half-life of greater than 48 h (28), is inhibited in Sindbis virus-infected BHK cells (Fig. 2), indicating that most of the BiP

molecules which associate with maturing E1 are produced prior to infection. It is possible that other ER resident factors, functioning either alone or in concert with BiP, are also involved in maintaining the fidelity of E1 folding.

In comparison with E1, very little PE2 is usually recovered in association with BiP (Fig. 1). PE2 may simply not interact with BiP under normal circumstances, or BiP-PE2 interactions may be more labile and/or have much shorter half-lives than BiP-E1 complexes. Since BiP likely functions in the translocation of proteins across the ER membrane (8), encounters between BiP and nascent PE2 are probable (because PE2 is the first of the Sindbis structural proteins translocated into the ER [43]). When E1 folding is aberrant, significantly increased levels of PE2 become associated with BiP (Fig. 4). These BiP-PE2 interactions form in a delayed fashion during chases after E1 has begun to misfold or has failed to fold efficiently. These results suggest that nascent PE2 is stable prior to pairing with E1 for only a restricted period of time, after which unpaired PE2 becomes recognized by BiP. Furthermore, these results imply that the productive association between PE2 and E1 must occur within a limited time frame and only after E1 has completed certain folding steps modulated by BiP binding and release. This latter conclusion is supported by kinetic studies which indicate that the pairing of PE2 with E1 is delayed after their synthesis (Fig. 5). Whether PE2 associates with any host factor(s) during the interim before pairing with E1 remains to be answered. Preliminary results from coimmunoprecipitation experiments with antibodies specific for the ER resident chaperones grp94 and calnexin have indicated that neither of these proteins is involved in the normal maturation of E1 or PE2 (unpublished observations).

ACKNOWLEDGMENTS

This research was supported by grant AI14710 from the National Institutes of Health 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.

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