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The Transmembrane Domain of the Hepatitis C Virus E2 Glycoprotein Is Required for Correct Folding of the E1 Glycoprotein and Native Complex Formation

Janisha Patel, Arvind H. Patel, and John McLauchlan[1]

MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, United Kingdom

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Hepatitis C virus (HCV) encodes two glycoproteins, E1 and E2, that interact to form both native and aggregated complexes in tissue culture cells. In native complexes, E1 and E2 are associated by noncovalent interactions and such complexes are considered to constitute the authentic interactions between the proteins. By contrast, the proteins are linked by covalent, disulfide bonds in aggregated complexes. From studies with a mutant in which cysteine residues in E1 have been substituted with other amino acids, we show that E1 continues to associate with E2, although the migratory patterns of the proteins on gels are consistent with the formation of aggregated complexes. Therefore, such complexes can be stabilized by noncovalent as well as covalent interactions. To further examine the requirements for native complex formation, segments of foreign glycoproteins were linked to regions of E2. Our data provide direct evidence for the requirement of C-terminal sequences in E2 that contain the transmembrane domain to permit oxidation of E1 and assembly of a native complex. By contrast, native complexes and oxidized E1 are not found in the presence of chimeric proteins containing the E2 ectodomain. These data suggest that interaction of E1 with the E2 transmembrane domain is critical for native complex formation.

Key Words: hepatitis C virus; HCV; E1; E2; complex formation; transmembrane domain; oxidation.

INTRODUCTION

Hepatitis C virus (HCV) encodes two glycoproteins, E1 and E2, which are thought to constitute the envelope of the virus (Houghton, 1996). It is well established that E1 and E2 associate and two types of complex have been identified (Grakoui *et al.,* 1993; Ralston *et al.,* 1993; Matsuura *et al.,* 1994; Selby *et al.,* 1994). In one complex, termed the native form, the proteins interact through noncovalent, intermolecular interactions. The second complex is considered to form through aggregation of incorrectly folded glycoproteins that associate by covalent, disulfide links. In a recent study using dithiothreitol (DTT) *in situ* to disrupt disulfide bonds, we showed that E1 and E2 in aggregated complexes could be connected also by noncovalent interactions (Patel *et al.,* 1999). The contribution of either complex to virion formation is not known, although it is considered that native complexes contain authentic interactions between the glycoproteins.

Conformational antibodies (Deleersnyder *et al.,* 1997; Duvet *et al.,* 1998) and sedimentation on density gradients (Ralston *et al.,* 1993; Dubuisson and Rice, 1996; Young *et al.,* 1998) were previously employed to distinguish between both types of complex. In addition, migration under nonreducing, denaturing conditions on polyacrylamide gels was used to identify the complexes that accumulate in cells (Ralston *et al.,* 1993; Dubuisson and Rice, 1996; Patel *et al.,* 1999). Such studies revealed that E1 and E2 from native complexes migrate as monomers, whereas the proteins in aggregated complexes barely enter the resolving component of gels. The native complex forms more slowly than the aggregated form and this is accompanied by the appearance of E1 in an oxidized state (Dubuisson and Rice, 1996). Since oxidation of E2 precedes that of E1, it has been suggested that E2 is required for correct folding of E1 (Dubuisson and Rice, 1996; Michalak *et al.,* 1997).

E1 and E2 cannot be detected on the cell surface and are retained within the endoplasmic reticulum (ER) (Ralston *et al.,* 1993; Selby *et al.,* 1994; Dubuisson and Rice, 1996; Cocquerel *et al.,* 1998). For both proteins, there is no evidence for cycling between the Golgi apparatus and ER (Duvet *et al.,* 1998; Cocquerel *et al.,* 1999) and hence ER localization is consistent with a retention mechanism rather than a process of retrieval from post-ER compartments. Retention is mediated by hydrophobic, transmembrane sequences present at the C termini of the proteins (Cocquerel *et al.,* 1998, 1999; Flint and McKeating, 1999). In the case of E2, replacement of the transmembrane region with the corresponding domains from proteins normally transported to the cell surface can direct E2 to the plasma membrane (Cocquerel *et al.,* 1998; Lagging *et al.,* 1998). Hence, retention in the ER is not solely a consequence of aberrant E2 folding. The transmembrane

[1] To whom correspondence and reprint requests should be addressed. Fax: +44 (0)141 337 2236. E-mail: j.mclauchlan@vir.gla.ac.uk.

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domains also function as signal sequences to direct cleavage from the adjacent gene product and single-site mutations in the transmembrane sequences are sufficient to either reduce or abolish signal peptide function (Cocquerel *et al.,* 2000). Finally, there is evidence that removal or mutation of the transmembrane domains inhibits formation of native complexes between E1 and E2 (Selby *et al.,* 1994; Michalak *et al.,* 1997; Cocquerel *et al.,* 1998, 2000). Controversially, other reports previously showed that the ectodomains of E1 and E2 alone are sufficient for interaction between the proteins (Matsuura *et al.,* 1994; Hussy *et al.,* 1997; Yi *et al.,* 1997), although these could represent aggregated complexes (Michalak *et al.,* 1997).

Oxidation of E1 is impaired in the absence of E2, leading to the suggestion that E2 performs a "chaperone-like" function (Dubuisson and Rice, 1996; Michalak *et al.,* 1997). In this report, we extended these studies by comparing the properties of E1 in the presence and absence of E2, to establish criteria for assessing the conformational status of E1. Since disulfide bonds have been implicated in complexes formed by the HCV glycoproteins and are necessary for E1 oxidation, the ability of a mutant form of E1 that lacks cysteine residues to associate with E2 and form a native complex was examined. Finally, we directly tested whether the ecto- or transmembrane domains of E2 are sufficient for native complex formation by fusion with segments from foreign glycoproteins of viral origin.

RESULTS

Behavior of HCV E1 in the presence and absence of E2

In a previous report, it was shown that generation of an oxidized, monomeric form of E1 is dependent on the presence of E2 (Michalak *et al.,* 1997). Using the Semliki Forest virus (SFV) system to express the HCV glycoproteins in baby hamster kidney (BHK) cells, we reexamined this phenomenon. After electroporation with RNA from two constructs, pSFV/E1 and pSFV/E1E2p7 (Fig. 1A), cells were radiolabeled and immunoprecipitations were performed on the resultant cell extracts using an E1-specific monoclonal antibody (MAb), AP21.010. Under nonreducing electrophoresis conditions, E1 and E2 were detected following immunoprecipitation with the extract from pSFV/E1E2p7-electroporated cells, indicating that they formed a native complex (Fig. 2A, lane 2). E1 migrated as a single species, whereas E2 was more diffuse. Western blot analysis using an E2-specific MAb confirmed the presence of E2 in the immunoprecipitated material (Fig. 2B, lane 2). The diffuse nature of E2 probably results from glycosylation of the protein, although the material may also contain both E2 and E2-p7 species as a result of the incomplete cleavage at the processing site between E2 and p7. This phenomenon was reported previously (Dubuisson *et al.,* 1994). For simplicity, the diffuse material will be referred to only as E2.

By contrast to the E1 species detected in the pSFV/E1E2p7 sample, E1 immunoprecipitated from the pSFV/E1 cell extract appeared as a series of regularly spaced bands, none of which comigrated with E1 from the pSFV/E1E2p7 sample (Fig. 2A, lane 1); upon endo H digestion of the pSFV/E1 sample, only a single E1 species was observed, indicating that the bands represent differentially glycosylated forms of the protein (data not shown). Examination of the samples under reducing conditions showed that the major E1 species from pSFV/E1E2p7 and pSFV/E1 cell extracts comigrated (Fig. 2C, lanes 1 and 2). Hence, the distinct mobilities in the nonreducing polyacrylamide gel were a consequence of different oxidation states of the protein in the presence and absence of E2. In addition, a series of bands of lesser mobility than E1, none of which comigrated with E2, were observed only with the pSFV/E1 sample in the reducing gel (Fig. 2C, lanes 1 and 2); these could be detected by Western blot analysis using an E1-specific antiserum (data not shown). Based on their apparent molecular weights, the species of >50 kDa match the predicted sizes of dimers, trimers, etc., of E1. Therefore, it was concluded that the higher-molecular-weight proteins represent oligomeric forms of E1. The oligomers presumably arise from disruption by the reducing agent of the aggregated material, which barely enters the resolving component of the nonreducing gel (Fig. 2A). Hence, disulfide links exist between E1 molecules in the aggregate; however, the appearance of oligomeric forms under reducing, denaturing conditions indicates that strong noncovalent intermolecular interactions also occur.

To further address the oxidation state of E1 produced in the presence of E2 and the role of disulfide bonds in the formation of E1 oligomers, two plasmids, pSFV/E1^{-cys} and pSFV/E1^{-cys}E2p7 expressing E1 alone and linked to E2, respectively, were made in which all eight of the cysteine residues in E1 had been altered to serine or alanine (Fig. 3A). The cysteine residue at position 207 was substituted with an alanine, since introduction of a serine at this position would create a novel predicted glycosylation site in E1. Apart from the cysteine substitutions, pSFV/E1^{-cys} and pSFV/E1^{-cys}E2p7 were identical to pSFV/E1 and pSFV/E1E2p7, respectively (Fig. 1A). Radiolabeled proteins from cell extracts electroporated with RNA from these constructs were assayed by immunoprecipitation with anti-E1 antiserum R528 and precipitated material analyzed by polyacrylamide gel electrophoresis. Under nonreducing conditions, the monomeric forms of cys$^-$ E1 protein generated by either of these constructs comigrated with the E1 species made in the absence of E2 but not with E1 synthesized in the presence of E2 (Fig. 3B; compare lanes 2 and 3 with lanes 1 and 4). Since cys$^-$ E1 protein cannot form intramolecular

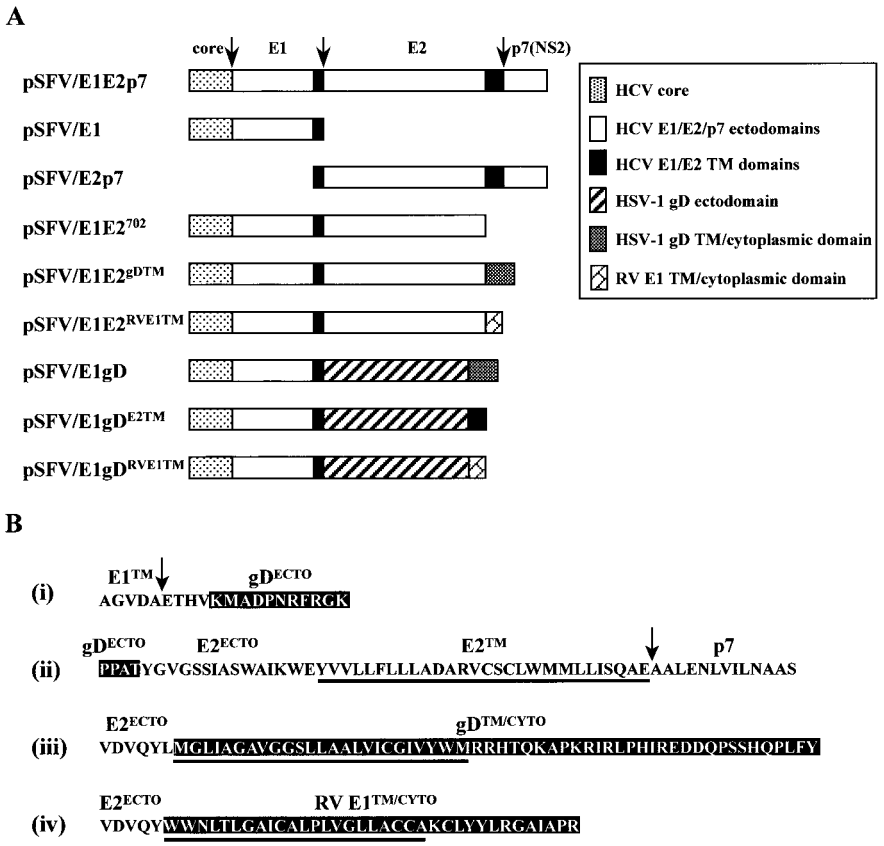

FIG. 1. (A) Schematic of SFV constructs. In all constructs except for pSFV/E2p7, the HCV E1 sequences were preceded by a segment of HCV core consisting of residues 1–65 linked to residues 169–191 that were used to direct translocation and correct processing of E1 and E2 in the ER. The C-terminal amino acid in constructs pSFV/E1E2p7 and pSFV/E2p7 was HCV residue 837, which was immediately followed by a stop codon; therefore, these plasmids contained the N-terminal region of NS2 as well as those of the glycoproteins and p7. The methionine residue at position 345 in the E1 transmembrane domain was used to initiate translation in plasmid pSFV/E2p7. The C termini of the coding sequences in pSFV/E1 and pSFV/E1E2^{702} were residues 387 and 702, respectively, which were immediately followed by a stop codon. In pSFV/E1E2^{gDTM} and pSFV/E1E2^{RVE1TM}, the HSV-1 gD and rubella virus (RV) E1 transmembrane/cytoplasmic sequences were linked to residues 702 and 701, respectively, of HCV E2. The predicted ectodomain of HSV-1 gD (amino acid residues 11–312; McGeoch *et al.,* 1988) was linked directly to HCV residue 387 in plasmids pSFV/E1gD, pSFV/E1gD^{E2TM}, and pSFV/E1gD^{RVE1TM}. The positions of cleavage sites that generate the mature HCV proteins are arrowed. (B) Amino acid sequences at the junctions of chimeric proteins. Sequences are shown for: (i) the junction between HCV E2 (ending at residue 387) and the ectodomain of HSV-1 gD (beginning at residue 11) in plasmids pSFV/E1gD, pSFV/E1gD^{E2TM}, and pSFV/E1gD^{RVE1TM}; (ii) the junction in pSFV/E1gD^{E2TM} between the ectodomain of HSV-1 gD (ending at residue 312) and residues 703–758 of the HCV polyprotein that contain the E2 transmembrane domain flanked by E2 ectodomain and p7 sequences; (iii) the junction between the ectodomain of HCV E2 (ending at residue 702) and the transmembrane/cytoplasmic sequences of HSV-1 gD (residues 316–370) in pSFV/E1E2^{gDTM}; and (iv) the junction between the ectodomain of HCV E2 (ending at residue 701) and the transmembrane/cytoplasmic sequences of rubella virus E1 (Hobman *et al.,* 1997). The HSV-1 and rubella virus residues are shown in shaded boxes and the predicted transmembrane regions for HCV E2, HSV-1 gD, and rubella virus E1 are underlined. The cleavage sites between HCV E1/E2 (i) and E2/p7 (ii) are arrowed.

disulfide bonds, this confirms that E1 associated with E2 in a native complex is in an oxidized state. Interestingly, a reduction in the amount of monomeric E2 that coprecipitated with the cys$^-$ E1 protein as compared to the wild-type form of the protein was evident in the nonreducing gel, although comparable amounts of E2 were present on reduction of the samples (compare Figs. 3B and 3C, lanes 1 and 2). Similar data were obtained when proteins were immunoprecipitated with the anti-E2 MAb ALP98 (Figs. 3D and 3E, lanes 1 and 2). Thus, removal of the cysteine residues in E1 does not prevent complex formation with E2, although there is an increase in the amount of E2 in such complexes that is in the form of an aggregate. We also noted in the nonreducing gel that oligomeric forms of cys$^-$ E1 were generated in the absence of E2 (Fig. 3B, lane 3). Oligomers continued to exist under reducing conditions, indicating that intermolecular disulfide bond formation is not essential for oligomerization, although the abundance of these products was reduced compared with that of wild-type E1 protein (Fig. 3C, lanes 3 and 4). This could result from reduced aggregation because of the inability of cys$^-$ E1 protein to form intermolecular disulfide bonds. In addition, oligomers identified for cys$^-$ E1 were slightly larger in size than those produced by wild-type protein (Fig. 3C, lanes 3 and 4). The size differences probably result from dif-

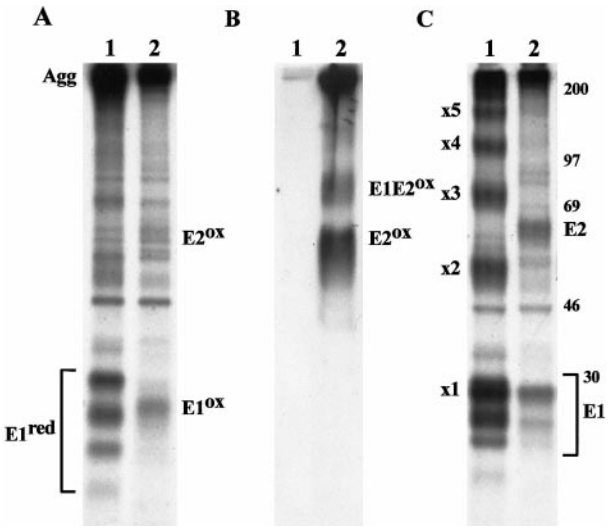

FIG. 2. Oxidation state of E1 expressed in the presence and absence of E2. Cells were electroporated with pSFV/E1E2p7 and pSFV/E1 RNAs and radiolabeled between 4.5 and 16 h after electroporation. After harvesting, immunoprecipitations were performed on the crude extracts with E1-specific MAb AP21.010. Eluted material was analyzed under nonreducing (A and B) and reducing (C) electrophoresis conditions. In (A) and (C), lane 1 shows precipitated material derived from cells electroporated with pSFV/E1 RNA and lane 2 contains precipitates from cells electroporated with pSFV/E1E2p7 RNA. (B) Western blot analysis using anti-E2 MAb ALP98 of the immunoprecipitated samples shown in (A) and (C). The positions of aggregated material (Agg), oxidized (ox), and reduced (red) E1 and E2 and oxidized, uncleaved E1E2 protein are indicated. Oligomers of E1 (x2, x3, etc.) also are shown.

ferential glycosylation of E1 in the oligomers since the endo H-digested oligomers of wild-type and cys$^-$ E1 comigrated (data not shown). The above-noted data in relation to E1 oxidation and oligomerization were reproducible over several experiments and therefore establish criteria to assess the behavior of E1 in the presence and absence of E2.

The ectodomain of E2 is not sufficient to prevent oligomerization of E1

The C-terminal hydrophobic sequences of E2 that contain the transmembrane domain were previously implicated in complex formation (Selby *et al.,* 1994; Michalak *et al.,* 1997; Cocquerel *et al.,* 1998, 2000), although other reports suggest that the ectodomain contributes to E1–E2 association (Matsuura *et al.,* 1994; Hussy *et al.,* 1997; Yi *et al.,* 1997). Therefore, we analyzed the behavior of E1 expressed along with E2 truncated at residue 702 and which, therefore, lacks E2 transmembrane domain sequences (Fig. 1A). Immunoprecipitations with the E2-specific MAb ALP98 revealed that oxidized E1 was present under nonreducing electrophoretic conditions on expression with full-length E2 but not with the truncated form of the protein (Fig. 4A, lanes 1 and 2). However, in a reducing gel, E1 could be detected along with truncated E2 (Fig. 4A, lanes 4 and 5). This indicates that E1–E2 interactions, which generate native complexes, are reduced to undetectable levels on removal of sequences containing the E2 transmembrane segment.

From a corresponding set of immunoprecipitations with the E1-specific antibody AP21.010, E1 made in the presence of truncated E2 gave a pattern of oligomerization on reducing gels which was identical to that found with E1 expressed alone (Fig. 4B, lanes 4 and 6). The dimeric form of E1 has a very similar apparent molecular weight to the truncated form of E2. Western blot analysis confirmed that the band representing dimers of E1 in Fig. 4B (lane 6) also contained truncated E2 (data not shown). Thus, as well as forming oligomers, some E1 could associate with truncated E2 as was observed with immunoprecipitations using the E2 MAb (Fig. 4A, lane 5). By contrast, E1 expressed along with full-length E2 showed no evidence of oligomers (Fig. 4B, lane 5). Under nonreducing conditions, monomeric E1 protein expressed in the presence of truncated E2 could be detected but it gave a complex pattern that was a combination of reduced and oxidized forms (Fig. 4B; compare lane 3 with lanes 1 and 2). We conclude that on expression with a truncated form of E2 that lacks the transmembrane domain, the majority of E1 exists in an aggregated state and only a small proportion can achieve an oxidized conformation.

The E2 transmembrane domain fused to the ectodomain of a heterologous glycoprotein allows E1 oxidation and prevents homo-oligomerization

In light of the above results, we tested whether the E2 transmembrane domain could affect the behavior of E1. A series of chimeric constructs were created in which the ecto- and transmembrane domains of E2 were substituted with corresponding regions of the herpes simplex virus type 1 (HSV-1) gD glycoprotein, an envelope component of virions (Figs. 1A and 1B) (McGeoch *et al.,* 1988). In addition, we used the transmembrane/cytoplasmic domain from rubella virus E1, a known ER retention signal (Hobman *et al.,* 1997), to replace the region containing the transmembrane domain in E2 for additional control experiments (Figs. 1A and 1B). In immunolocalization studies, we found that wild-type E2 could not be detected on the cell surface, in agreement with previous findings (data not shown) (Cocquerel *et al.,* 1998). Linking either the rubella virus E1 or HSV-1 gD transmembrane/cytoplasmic domains to the E2 ectodomain directed barely detectable amounts of E2 to the cell surface and only small quantities of the E2 chimeras were resistant to endo H digestion (data not shown).

Based on these assays, we concluded that the bulk of the chimeric proteins containing the E2 ectodomain remain in the ER. Cells electroporated with RNA from a construct encoding a polyprotein consisting of HCV E1

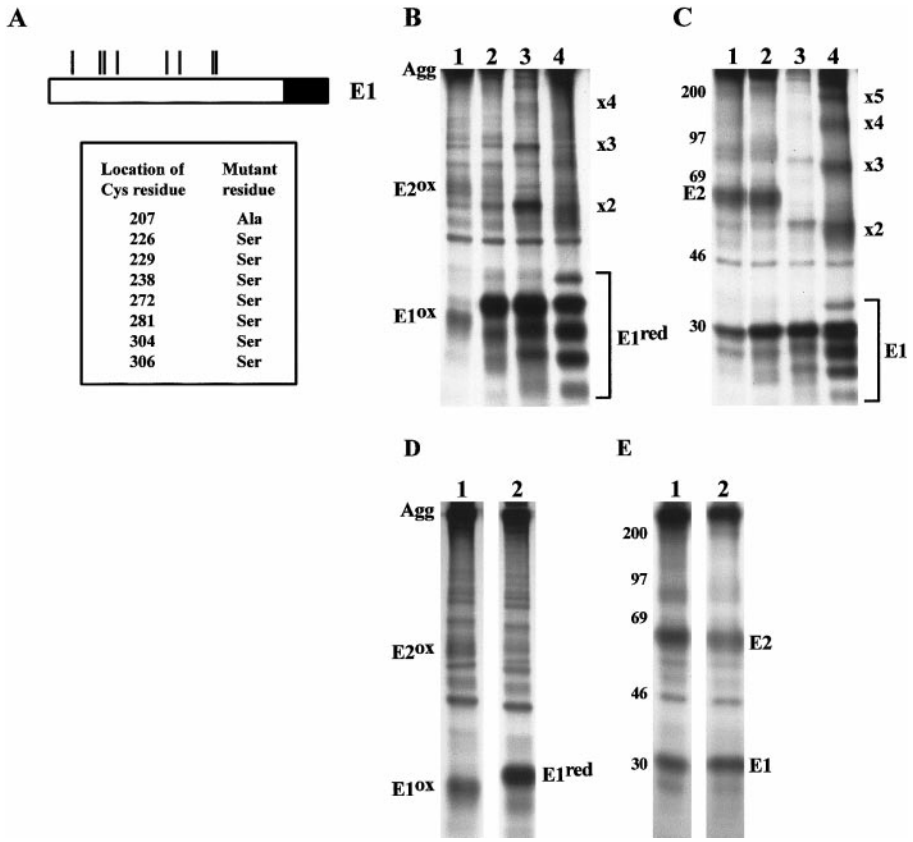

FIG. 3. The effect of substituting cysteine residues in E1 on complex formation. (A) Location and substitution of cysteine residues in E1. Vertical bars indicate the positions of cysteine residues in E1 and the shaded area represents the transmembrane domain of the protein. (B–E) Cells were electroporated with RNA derived from pSFV/E1E2p7 (lanes 1), pSFV/E1^{-cys}E2p7 (lanes 2), pSFV/E1^{-cys} [lanes 3, (B) and (C) only], and pSFV/E1 [lanes 4, (B) and (C) only]. After cells were radiolabeled, crude extracts were subjected to immunoprecipitation with E1-specific antiserum, R528 (B and C) and the E2 MAb, ALP98 (D and E). Precipitates were analyzed under nonreducing (B and D) and reducing (C and E) electrophoresis conditions. Positions of the aggregated material (Agg), oxidized (ox), and reduced (red) forms of E1 and E2, and oligomeric forms of E1 (x2, x3, etc.) are shown.

linked to the HSV-1 gD coding sequences showed surface expression of the HSV-1 glycoprotein but not E1 (Fig. 5A, panels *i* and *v*); permeabilization of cells, however, did reveal intracellular E1 protein (Fig. 5A, panel *vi*). Replacing the transmembrane and cytoplasmic regions of gD with a segment containing the transmembrane domain of E2 (gD^{E2TM} protein; Figs. 1A and 1B) dramatically reduced the abundance of gD at the plasma membrane, with barely detectable amounts of protein observed at the cell surface (Fig. 5A, panel *iii*). From Western blot analysis, two gD species could be detected in pSFV/E1gD cell extracts (labeled in Fig. 5B, lane 4) that were indistinguishable in size from the products found in HSV-1-infected cells (data not shown). Therefore, we assume that gD is produced by proteolytic processing from the E1-gD polyprotein by cleavage at the end of the E1 sequences and is posttranslationally modified to give proteins that are normally generated during HSV-1 infection. The higher-molecular-weight product, representing the mature form of the protein that is both N- and O-glycosylated (labeled ● in Fig. 5B, lane 4) (Cohen *et al.,* 1983; Johnson and Spear, 1983; Matthews *et al.,* 1983; Serafini *et al.,* 1988), was resistant to endo H digestion (Fig. 5B, lane 5). By contrast, the lower-molecular-weight product (labeled ○ in Fig. 5B, lane 4), indicative of glycosylated but immature gD, was partially sensitive (Fig. 5B, lane 5). Only one major species was detected for gD^{E2TM} protein which was sensitive to endo H digestion (Fig. 5B, lanes 1 and 2). Treatment with PNGase F also showed that glycosidase-digested gD was of higher molecular weight than gD^{E2TM} (Fig. 5B, lanes 3 and 6). Since O-glycosylation of gD occurs in the Golgi apparatus (Johnson and Spear, 1983) and the transmembrane segment of E2 is only slightly shorter than the transmembrane/cytoplasmic sequences of gD (Fig. 1B), we assume that the lower molecular weight for gD^{E2TM} primarily results from lack of O-glycosylation. Hence, in agreement with other studies (Cocquerel *et al.,* 1998; Duvet *et al.,* 1998), our immunofluorescence and glycosidase digestion data demonstrate that the segment containing the E2 transmembrane domain is sufficient to retain in the ER a foreign glycoprotein that normally exits the organelle.

To examine the behavior of E1 on expression with the chimeric proteins, immunoprecipitations were performed with antibodies specific for E2, gD, and E1. Immunopre-

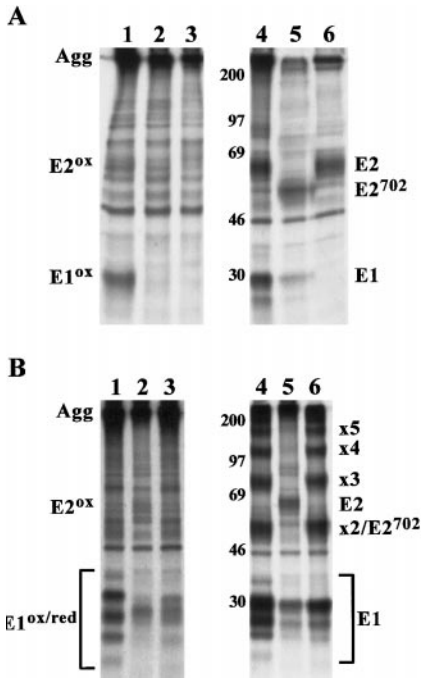

FIG. 4. Examination of complex formation between E1 and truncated E2 protein. Cells were electroporated with RNA and radiolabeled. Crude extracts derived from these cells were subjected to immunoprecipitation with the E2-specific MAb, ALP98 (A) and E1-specific MAb, AP21.010 (B). Precipitated material was analyzed under nonreducing (lanes 1–3) and reducing (lanes 4–6) electrophoresis conditions. Samples were pSFV/E1E2p7 [lanes 1 and 4 in (A), lanes 2 and 5 in (B)], pSFV/E1E2^{702} [lanes 2 and 5 in (A), lanes 3 and 6 in (B)], pSFV/E2p7 [lanes 3 and 6 in (A)], and pSFV/E1 [lanes 1 and 4 in (B)]. The bands corresponding to aggregated material (Agg), E2, truncated E2 (E2^{702}), E1, and E1 oligomers are indicated.

cipitation with anti-E2 MAb ALP98 revealed that oxidized E1 was not associated with the E2 ectodomain linked to either gD or rubella virus transmembrane/cytoplasmic sequences (Fig. 6A, lanes 1–3). Under reducing conditions, small quantities of E1 could be detected that had interacted with the E2 chimeras (Fig. 6A, lanes 7 and 8). Similar results were obtained with HCV E1 and HSV-1 gD proteins generated from the E1-gD polyprotein using the gD antibody 4846 (Fig. 6A, lanes 5 and 10), suggesting that there may be some nonspecific interaction between the HCV and HSV-1 proteins. Notably, an oxidized form of E1 was efficiently coprecipitated by the gD antibody along with oxidized gD^{E2TM} protein (Fig. 6A, lane 4). From immunoprecipitations with an E1-specific antiserum R528, the E1 species precipitated from the pSFV/E1gD^{E2TM} cell extract was indicative of an oxidized conformation, although it did not precisely comigrate with the oxidized form of E1 synthesized in the presence of E2 (Fig. 6B, lanes 2 and 3). A slight difference in the mobilities of E1 expressed with E2 and gD^{E2TM} proteins also is evident in Fig. 6A (compare lanes 1 and 4). This may suggest subtle changes to the folding of E1 in the presence of the chimeric protein. Under reducing conditions, we did observe greater heterogeneity in the size of E1 species produced by pSFV/E1gD^{E2TM} (Figs. 6A and 6B, lane 9).

A similar phenomenon was found with E1 made by other constructs (Fig. 6B, lanes 10–12) and may indicate altered glycosylation or modification to E1. Again, this may account for the differences in mobility found under nonreducing conditions for oxidized E1 produced by pSFV/E1E2p7 and pSFV/E1gD^{E2TM}. As was the case with the gD antibody, gD^{E2TM} did coprecipitate with E1 using anti-E1 antiserum R528 and could be detected under both reducing and nonreducing conditions (Fig. 6B, lanes 3 and 9). In construct pSFV/E1gD^{E2TM}, there are 13 residues of p7 downstream from the E2 transmembrane segment that were included to allow processing at the E2/p7 cleavage site, which normally occurs during maturation of the proteins (Fig. 1B). Since processing at this site may not be 100% efficient, it is possible that some of the gD^{E2TM} protein contains p7 residues that may contribute to E1 folding. To address this possibility, another construct was made that was identical to pSFV/E1gD^{E2TM}, except that it lacked the p7 sequences; results with this construct were identical to those shown for pSFV/E1gD^{E2TM} in Fig. 6 (data not shown). Therefore, E1 can efficiently form a complex with a chimeric protein that contains only HCV E2 residues 703–745, which encompass the transmembrane domain, and the E1 protein in such complexes is oxidized. Further data with the anti-E1 antiserum revealed that only faint quantities of E1 were detected with the chimeras containing the E2 ectodomain under nonreducing conditions and there was no evidence of chimeric forms of E2 (Fig. 6B, lanes 4–6). On a reducing gel, oligomeric forms of E1 were apparent on expression with the E2 ectodomain chimeric proteins and gD (Fig. 6B; compare lanes 10–12 with lane 7). Under these conditions, we did note the appearance of bands corresponding in size to the chimeric proteins containing the ectodomain of E2 (indicated as ■ and □ in Fig. 6B, lanes 10 and 11). This again suggests that, although the E2 ectodomain can associate with E1, the resultant complexes represent aggregates. By contrast, no oligomers of E1 were found with the gD ectodomain linked to the E2 transmembrane segment (Fig. 6B, lane 9), further indicating that E1 aggregates to a far lesser extent in the presence of this chimeric protein.

To verify that oxidation and lack of aggregation of E1 were a direct consequence of the presence of the E2 transmembrane segment, the behavior of E1 was examined using another construct which was identical to pSFV/E1gD^{E2TM}, except that the gD ectodomain was linked to the transmembrane/cytoplasmic sequences of the rubella virus E1 glycoprotein (Fig. 1A). In immunolocalization studies, the gD/RVE1 chimera was not present on the surface of expressing cells and had a distribution coincident with that of E1 (data not

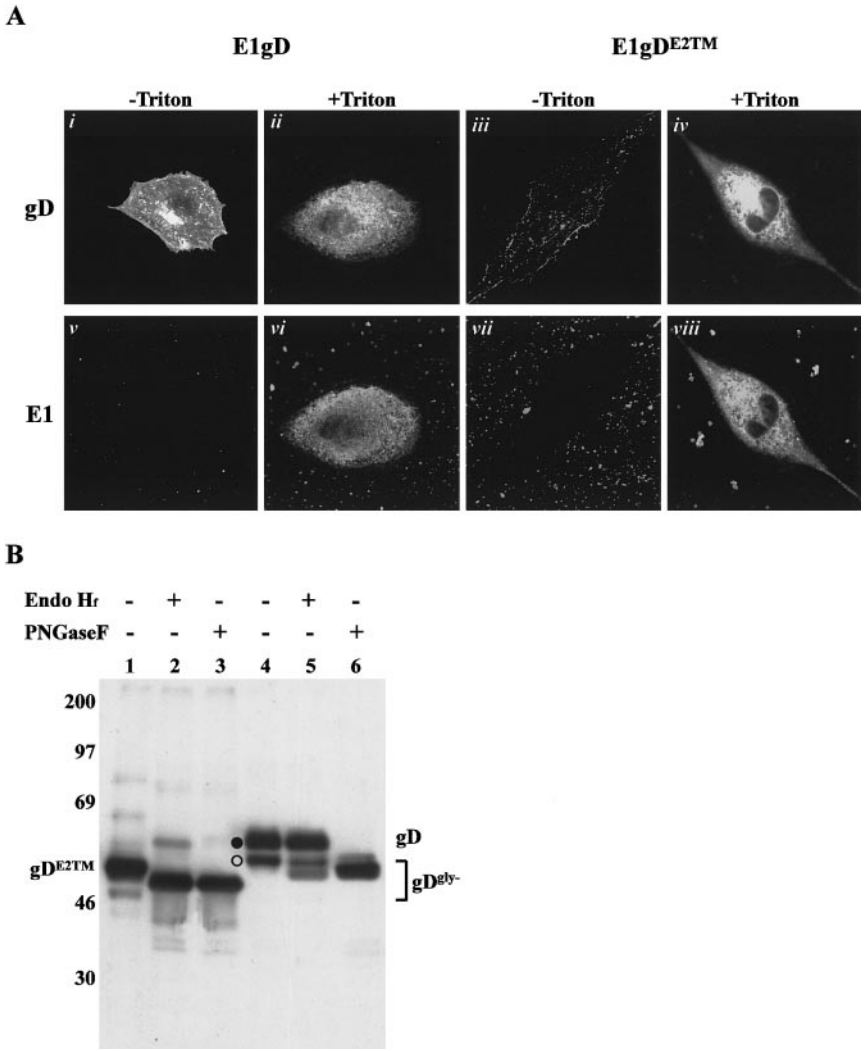

FIG. 5. Intracellular localization of E1, gD, and gD^{E2TM} proteins. (A) Immunolocalization of proteins. Cells electroporated with RNA transcripts were fixed 12 h after electroporation with paraformaldehyde (PFA; panels *i, iii, v,* and *vii*) or PFA containing 0.1% Triton X-100 (panels *ii, iv, vi,* and *viii*). Cells were processed for indirect immunofluorescence using HSV-1 gD-specific MAb 4846 (panels *i–iv*) and anti-E1 antiserum R528 (panels *v–viii*). Constructs from which RNA was synthesized were pSFV/E1gD (panels *i, ii, v,* and *vi*) and pSFV/E1gD^{E2TM} (panels *iii, iv, vii,* and *viii*). (B) Endo H sensitivity of HSV-1 gD proteins. Cells were lysed 12 h postelectroporation and portions of the crude extracts were treated with endo H and PNGase F. Following electrophoresis under reducing conditions, Western blot analysis was performed using gD-specific MAb 4846. Samples were pSFV/E1gD^{E2TM} (lanes 1–3) and pSFV/E1gD (lanes 4–6). Positions of glycosylated and deglycosylated forms of gD proteins are indicated. Mature (●) and immature forms (○) of gD referred to in the text are labeled.

shown). We found that HCV E1, expressed from this construct, gave a pattern of bands identical to that for E1 expressed alone on examination under nonreducing electrophoretic conditions (Fig. 7; compare lane 2 with lanes 1 and 3). Although some oxidized E1 may be present in these species, the similarities in the E1 patterns for the pSFV/E1gD^{RVE1TM} and pSFV/E1 samples suggest that most of E1 produced by pSFV/E1gD^{RVE1TM} does not contain intramolecular covalent bonds. Moreover, oligomers of E1 were detected in reducing gels for pSFV/E1gD^{RVE1TM} (Fig. 7, lane 5). These data confirm that the E2 transmembrane segment has a critical role in the folding of E1, which is not simply a result of its ER retention properties.

DISCUSSION

Our studies further characterized the properties of the HCV E1 glycoprotein by comparing its behavior in the presence and absence of E2. In the absence of E2, E1 forms aggregates that are disrupted by reducing agents, to generate multimeric forms of the protein that can be identified upon gel electrophoresis. The existence of oligomers under reducing conditions indicates that noncovalent, intermolecular interactions can occur between E1 molecules. Covalent interactions also appear to contribute to E1–E1 association since the abundance of oligomers composed of E1 protein lacking any cysteine residues is reduced. Thus, the interactions between E1

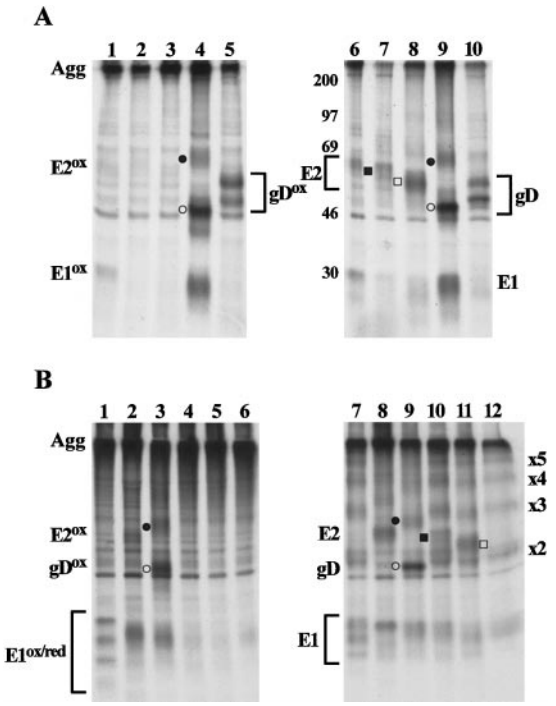

FIG. 6. Examination of complex formation by chimeric E2 and gD proteins with E1. Cells, electroporated with RNA, were radiolabeled. Following cell lysis, crude extracts were subjected to immunoprecipitation using E2-specific MAb ALP98 (lanes 1–3 and 6–8) and gD-specific MAb 4846 (lanes 4, 5, 9, and 10) in (A). In (B), immunoprecipitations were performed with E1 antiserum R528. Precipitated material was examined under nonreducing [lanes 1–5 in (A), lanes 1–6 in (B)] and reducing [lanes 6–10 in (A), lanes 7–12 in (B)] electrophoresis conditions. Samples in (A) were derived from pSFV/E1E2p7 (lanes 1 and 6), pSFV/E1E2^{gDTM} (lanes 2 and 7), pSFV/E1E2^{RVE1TM} (lanes 3 and 8), pSFV/E1gD^{E2TM} (lanes 4 and 9), and pSFV/E1gD (lanes 5 and 10). In (B), samples were derived from pSFV/E1 (lanes 1 and 7), pSFV/E1E2p7 (lanes 2 and 8), pSFV/E1gD^{E2TM} (lanes 3 and 9), pSFV/E1E2^{gDTM} (lanes 4 and 10), pSFV/E1E2^{RVE1TM} (lanes 5 and 11), and pSFV/E1gD (lanes 6 and 12). The bands corresponding to aggregated material (Agg), oxidized and reduced forms of E1, E2, and gD, and E1 oligomers are indicated. Uncleaved E1-gD^{E2TM} polyprotein (●) and mature gD^{E2TM} (○) are labelled as are the E2^{gDTM} (■) and E2^{RVE1TM} (□) chimeric forms of E2.

proteins may be covalent or noncovalent or, indeed, a combination of both. By comparing the migratory patterns of native E1 and a mutant form that lacks cysteine residues, we also found that monomeric E1 synthesized in the absence of E2 remained in a reduced state. This contrasts with E1 made in the presence of E2, wherein intramolecular disulfide bonds form and an oxidized state is attained. These data confirm previous observations on the requirement of E2 for intramolecular disulfide bond formation within individual E1 molecules (Michalak *et al.,* 1997). The appearance of oxidized E1 and native E1–E2 complexes coincide and thus it is assumed that such forms of E1 are correctly folded (Dubuisson and Rice, 1996; Deleersnyder *et al.,* 1997; Michalak *et al.,* 1997). The behavior of the HCV glycoproteins is similar to that for the glycoproteins E1 and p62, encoded by SFV, that interact to form a heteromeric complex (Ziemiecki *et al.,* 1980). In the absence of p62, the majority of SFV E1 is in an oligomeric, aggregated state with very little oxidized protein detected (Andersson *et al.,* 1997). By contrast, SFV E1 has a less critical role in the folding of p62. Thus, it has been suggested that p62 assists E1 folding through the formation of a p62/E1 heteromeric complex (Andersson *et al.,* 1997) and therefore may have a function analogous to that for HCV E2.

Disrupting the ability of HCV E1 to form intramolecular disulfide bonds by mutating all of the cysteine residues does not prevent complex formation with E2 but E2 is aggregated. Therefore, within aggregates, E1 and E2 can associate through noncovalent interactions and their interaction need not be stabilized by intermolecular covalent bonds. This agrees with our previous findings in which the disulfide bonds in aggregated complexes could be disrupted in cells by DTT, although E1 and E2 continued to associate (Patel *et al.,* 1999). Since native complexes also are stabilized by noncovalent interactions, this highlights the difficulty with verifying the nature of E1–E2 complexes that are produced in mammalian systems that, to date, do not efficiently generate virus particles. Hence, in the absence of any functional assay for E1, we employed the existence of nonoligomeric, oxidized forms of the protein as parameters for authentic folding.

Based on the above-mentioned parameters, removing the C-terminal sequences from E2 including the transmembrane region reduces but does not prevent association with E1. However, the resultant complexes are aggregated. Fusion of the transmembrane/cytoplasmic regions from HSV-1 gD and rubella virus E1 to the HCV E2 ectodomain did not restore native forms of E1 and E2. This agrees with studies published previously, in which replacing the E2 transmembrane domain with alternative

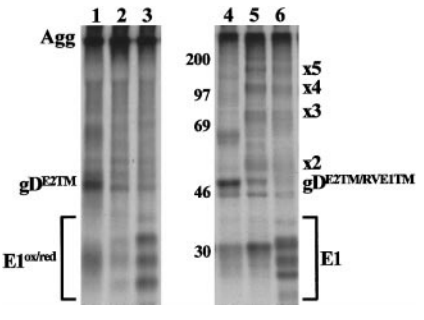

FIG. 7. Examination of the behavior of E1 expressed along with gD fused to the rubella virus E1 transmembrane/cytoplasmic domain. Cells were electroporated with RNA and radiolabeled, and crude extracts were subjected to immunoprecipitation using anti-E1 antiserum R528. Precipitates were examined under nonreducing (lanes 1–3) and reducing (lanes 4–6) electrophoresis conditions. Samples were pSFV/E1gD^{E2TM} (lanes 1 and 4), pSFV/E1gD^{RVE1TM} (lanes 2 and 5), and pSFV/E1 (lanes 3 and 6). Positions of aggregated material (Agg), chimeric gD proteins (gD^{E2TM} and gD^{RVE1TM}), E1, and oligomerized forms of the protein are indicated.

sequences abolishes native complex formation (Selby *et al.,* 1994; Michalak *et al.,* 1997; Cocquerel *et al.,* 1998). The transmembrane/cytoplasmic domain from rubella virus E1 behaves as an ER retention signal (Hobman *et al.,* 1997) and thus has similar functional characteristics to the E2 transmembrane segment. Therefore, retention of E2 in the ER alone is not sufficient to permit native complex formation. Significantly, fusion of sequences containing the E2 transmembrane domain to the ectodomain of HSV-1 gD generated complexes in which nonaggregated gD^{E2TM} chimeric protein could be detected and E1 was in an oxidized state. Moreover, oligomerization of E1 was not detected. Thus, the complex formed by E1 and gD^{E2TM} has features consistent with those of a native complex. Processing at the proteolytic site between E2 and p7 was not necessary for formation of the complex since identical data could be obtained using a fragment of E2 that did not contain p7 sequences. Therefore, our data formally demonstrate that a C-terminal segment of E2 composed predominantly of the transmembrane domain is not only important for E1–E2 complex formation but also critical for correct folding of E1.

As in the case of the interactions between SFV p62 and E1, it has been suggested that E2 has a "chaperone-like" function to facilitate oxidation and folding of E1 (Michalak *et al.,* 1997). The ability of E1 to oxidize with a chimeric protein consisting of the HSV-1 gD ectodomain fused to the E2 transmembrane sequences suggests that the function of the HCV E2 ectodomain in E1 folding can be substituted by the equivalent region from a foreign glycoprotein. Although it remains possible that there are specific interactions between E1 and the E2 ectodomain, these do not appear to be essential to prevent aggregation of E1. We propose that interaction between the E2 transmembrane domain and E1 is a key step in the folding of E1 but that the role of the ectodomain may be less specific. It is possible that upon interaction of the E2 transmembrane domain with E1, the E2 ectodomain acts as a buffer to prevent association of E1 molecules within close proximity to each other. Thus, the roles of the transmembrane and ectodomains of E2 during folding of E1 are distinct.

Another feature that we observed was the accumulation of incompletely glycosylated forms of E1 if no glycoprotein was linked downstream of the E1 coding sequence (e.g., Fig. 2C, lanes 1 and 2). Appearance of these species was not dependent on interaction of E1 with its linked partner since they were less apparent on expression of a polyprotein containing E1 fused to glycoproteins with which it did not interact efficiently (see Fig. 6B, lanes 7 and 12; Fig. 7, lanes 5 and 6). An identical series of bands corresponding to different E1 glycoforms also has been identified upon reversing the order of E1 and E2 on the polyprotein (Cocquerel *et al.,* 2000). Similar to our construct that expressed E1 alone, there were no sequences beyond the C terminus of the glycoprotein. This possibly suggests that processes at the E1/E2 cleavage site are important for efficient production of glycosylated E1. Previous studies showed that glycosylation of E1 at position 325 does not occur as a consequence of a proline residue adjacent to the predicted site (Meunier *et al.,* 1999). Our data suggest that there may be additional interactions between E1 and the cellular machinery, which also are required for efficient glycosylation at the other sites in E1.

In conclusion, we demonstrated that a short region of E2 containing the transmembrane domain is essential for folding of E1 and native complex formation. Attempts to identify the region in E1 to which the E2 transmembrane region binds have not been successful since mutations within the protein result in enhanced aggregation (data not shown). Additional studies and other approaches are therefore necessary to examine the sequences in E1 that are critical for native complex formation. Such analysis will shed further light on the complexes formed by the HCV glycoproteins and the processes involved in virion morphogenesis.

MATERIALS AND METHODS

Cell culture

BHK C13 cells were grown and maintained in Glasgow minimal Eagle's medium (GMEM) supplemented with 10% newborn calf serum (NCS), 4% tryptose phosphate broth, and 100 IU/ml penicillin/streptomycin (ETC10).

Construction of plasmids

A plasmid, pCV-H77c (kindly supplied by J. Bukh), carrying the infectious full-length cDNA clone of HCV strain H77 (Yanagi *et al.,* 1997), provided the DNA fragments for HCV sequences. The HSV-1 gD coding sequence was amplified by PCR from a clone, pET28/Bamj, provided by V. Preston. Details of the regions used in this study to express E1, E2, and chimeric proteins are given in Fig. 1. All fragments were initially cloned into vector pGEM-1 (Promega, UK) and then flanked by *Bgl*II sites. Recombinant SFV expression vectors were generated by introduction of *Bgl*II fragments containing coding sequences into the unique *Bam*HI site of pSFV1 (Liljestrom and Garoff, 1991). pSFV/E1 and $pSFV/E1^{-cys}$ were derived from pSFV/E1E2p7 and $pSFV/E1^{-cys}E2p7$, respectively. Constructs were made by standard methods using restriction enzyme fragments along with synthetic oligonucleotides to combine fragments with incompatible termini and for mutation of cysteine residues in E1. The E2 transmembrane and HSV-1 gD transmembrane/cytoplasmic domain coding sequences were amplified by PCR, whereas the rubella virus E1 transmembrane/cytoplasmic domain sequences were introduced using synthetic oligonucleotides. The nucleotide sequences of all regions that were modified as a consequence of cloning procedures were determined before insertion into pSFV1.

In vitro transcription and electroporation of SFV RNA into cells

Recombinant pSFV DNAs were linearized with *Spe*I and purified by phenol/chloroform extraction and ethanol precipitation. After pelleting, DNA was resuspended in 15 μl H_2O. *In vitro* transcription reactions were typically performed in a final volume of 50 μl as follows: NTP mix (1 mM ATP, UTP, CTP, 0.5 mM GTP), transcription buffer (40 mM Tris, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl), 1 mM $m^7G(5')ppp(5')G$ RNA cap analog, 1 mM DTT, 2 units/μl human placental ribonuclease inhibitor (RNasin), 2 units/μl SP6 RNA polymerase. A 1.5-μg sample of linearized template DNA was added to reactions which were incubated at 37°C for 1–2 h.

For electroporation, cells approaching confluency in a 160-cm^2 flask were removed by trypsin treatment, resuspended in 10 ml ETC10 and pelleted by centrifugation at 400 *g* for 5 min at room temperature. The cell pellet was resuspended in 30 ml PBS(A) and pelleted as before. BHK cells were finally resuspended in PBS(A) to a final concentration of $\sim 1 \times 10^7$ cells/ml. A 0.8-ml aliquot of resuspended cells was placed in an electroporation cuvette (0.4-cm gap; Bio-Rad, Richmond, CA) with 25 μl of recombinant pSFV RNA. The cell/RNA suspension was mixed by inversion and electroporated at 1.2 kV and 25 μF (Bio-Rad Gene Pulser II) at room temperature. The cells were pulsed twice with an inversion step between the pulses and then diluted 1/20 in ETC10. Thereafter, cells were seeded onto 35-mm tissue culture dishes or 13-mm coverslips and incubated at 37°C.

Metabolic labeling of proteins and production of cell lysates

Medium from cells incubated at 37°C for 4 h postelectroporation was replaced with GMEM containing one-fifth normal concentration of methionine and calf serum at a final concentration of 2% [GMEM (low met)]. Following further incubation at 37°C for 30 min, the medium was again replaced with GMEM (low met) containing 10 μCi/ml ^{35}S-methionine. Cells were radiolabeled for 12 h at 37°C before harvesting.

For harvesting, cells were washed twice with 1 ml ice-cold PBS, containing 20 mM *N*-ethylmaleimide (NEM), an alkylating agent that blocks free sulfhydryl groups on cysteine residues. Cells were lysed using 500 μl of immunoprecipitation buffer (25 mM Tris–HCl, pH 7.5, 300 mM NaCl, 1% Triton X-100, 20 mM NEM, 1 mM EDTA, 1 mM PMSF). Complete lysis was achieved by incubation on ice for 10 min and the cell debris was pelleted by centrifugation at 13,000 *g* for 10 min at room temperature.

Deglycosylation of proteins

Lysates from cells were denatured in 0.5% (w/v) SDS, 1% (v/v) β-mercaptoethanol at 100°C for 10 min. Sodium citrate was added to 50 mM followed by 2000 units of endo H or 1000 units of PNGase F. For PNGase F digestions 1% (v/v) NP-40 was added to the reaction mix following the denaturation step to inactivate SDS. The reactions were incubated at 37°C for 1 h.

Immunoprecipitation of proteins

Cell lysates were incubated at 4°C overnight in 1–5 μl of the appropriate antibody (diluted 1/500), followed by addition of 50 μl of equilibrated Protein A–Sepharose (Sigma, UK) and incubation at 4°C for a further 1 h. Antibodies used were AP21.010 (an anti-E1 MAb), R528 (an anti-E1 rabbit antiserum), ALP98 (an anti-E2 MAb) (Patel *et al.,* 1999), and 4846 (an anti-HSV-1 gD MAb) (A. Cross and H. Marsden, personal communication). E1 and E2 antibodies were raised against purified E1 and E2 expressed in mammalian cells that were derived from HCV strain Glasgow (Patel *et al.,* manuscript in preparation). Immune complexes attached to Sepharose were pelleted at 1000 *g* for 30 s and washed three times with immunoprecipitation buffer. Proteins bound to Sepharose were removed by addition of 200 mM Tris–HCl, pH 6.8, 0.5% SDS, 10% glycerol, and 0.01% bromphenol blue.

Polyacrylamide gel electrophoresis and Western blot analysis

Samples were prepared for electrophoresis by heating to 95°C. For electrophoresis under reducing conditions, DTT was added to samples to a final concentration of 20 mM prior to heating. Cooled samples were electrophoresed on 10 or 12% polyacrylamide gels cross-linked with 2.5% (w/w) *N,N′*-methylene bisacrylamide (Laemmli, 1970). Polypeptides were detected by autoradiography using XS-1 X-ray film (Kodak, Rochester, NY).

For Western blot analysis, proteins were separated on polyacrylamide gels and transferred to nitrocellulose membrane (Towbin *et al.,* 1979). After blocking with 5% milk (Marvel) in PBS, the membrane was incubated with an E1-specific antiserum (R528; diluted 1/1000) or E2 MAb (ALP98; diluted 1/500) for 4 h in PBS containing 1% BSA and 0.05% Tween 20. After washing, bound antibody was detected using a horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence (Amersham, UK).

Indirect immunofluorescence

Electroporated cells on coverslips were fixed in methanol and incubated for 20 min at −20°C. Alternatively, nonpermeabilized cells were prepared by fixing with 4% paraformaldehyde (PFA; Sigma) for 30 min at 4°C. Permeabilized cells were fixed under the same conditions in PFA containing 0.1% (v/v) Triton X-100. Rehydrated cells were examined for expression of the protein of interest using the relevant primary antibody (diluted 1/200) followed by visualization using goat anti-mouse (FITC-con-

jugated; Sigma) and goat anti-rabbit (Cy5-conjugated; Sigma) IgG (diluted 1/100).

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