The Proteolytic Cleavage of PE2 to Envelope Glycoprotein E2 Is Not Strictly Required for the Maturation of Sindbis Virus

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The ionophore monensin has been shown previously to block the maturation of Sindbis virus as well as prevent the cleavage of PE2 to E2 when applied to cells in high concentration. We found that a moderate dose of monensin reduced virus titer and inhibited the cleavage of PE2 to E2. Under these conditions, PE2 appeared on the cell surface in a form susceptible to lactoperoxidase-mediated iodination. This PE2 was incorporated into virions, replacing E2. PE2-containing virions had a normal PFU-to-particle ratio, cosedimented with normal virus, and retained a normal morphology when negatively stained preparations were examined by electron microscopy. We conclude that the cleavage of PE2 to form E2 is not an absolute prerequisite for virus maturation. Recently, Russell et al. have reached a similar conclusion (D. L. Russell, J. M. Dalrymple, and R. E. Johnston, J. Virol. 63:1619–1629, 1989).

Sindbis virus, a member of the family Togaviridae, is a structurally uncomplicated enveloped virus which matures by envelopment at the cell surface. Mature virions contain two membrane glycoproteins (E1 and E2) and an internally situated capsid protein, C. The structural proteins of the virus are translated as a large polyprotein which is cleaved to give C, E1, and pE2 (the precursor to E2) (26). The envelope precursors PE2 and E1 are translocated into the rough endoplasmic reticulum and are then transported through the Golgi vesicles to the cell membrane in a manner similar to other integral membrane proteins (10, 15). PE2 is unusual in that the hydrophobic leader sequence is not cleaved immediately upon translocation into the rough endoplasmic reticulum. En route (M. E. Knipe and D. T. Brown, Virology, in press) or on the cell surface (2, 14), PE2 is cleaved to form E2 and a small glycopeptide, E3.

Although PE2 cannot be detected on the cell surface by direct procedures, such as enzymatic radioiodination (13, 29), indirect evidence suggests that it may be present at the cell surface in a cryptic form (10, 25, 29). Studies employing antibodies specific for polypeptide E2 show that these antibodies can block the cleavage of PE2 to E2, suggesting that some important fraction of the total cleavage of PE2 to E2 occurs at the cell surface (2, 14). PE2 has been recovered in the plasma membrane fraction in membrane fractionation experiments (10, 25). The temperature-sensitive mutant ts20 has a single amino acid substitution in the E2 protein, and at the nonpermissive temperature, PE2 is not cleaved to E2 (18). However, virus protein is transported to the cell surface and binds nucleocapsid (3, 10, 19, 29).

All drugs that block the cleavage of PE2 to E2, such as monensin, cerulinen, and tunicamycin, also prevent maturation of virus. Similarly, mutants, such as ts20 and ts23 from the Pfiefferkorn set (4–6), block the cleavage of PE2 to E2 as well as virus maturation. PE2 has never been recovered in mature virus (13, 17, 26, 29). It is therefore generally assumed that cleavage of PE2 is a prerequisite for virus maturation.

The finding that enzymatic radiolabeling with lactoperoxidase and 125I does not label PE2 in cells infected with either wild-type virus or ts20, although the product E2 is extensively labeled by this procedure (13, 29), led us to suggest that a major change in the conformation of the PE2 or E2 protein accompanies proteolysis and that this conformational change was essential for establishing the functional potential of the protein (29). We also have found that dramatic changes in the conformation of E2 in mature virions can accompany changes in environmental pH (9). These observations suggest that the glycoproteins of alphaviruses may be delivered into mature virions as metastable structures. To determine if the achievement of this metastable conformation is dependent upon exposure to a particular pH during intracellular transport, we have examined the conformation of PE2 in the presence of agents which perturb the pH of intracellular compartments.

In this study, we have used the drug monensin, an ionophore that catalyzes the exchange of sodium ions for protons across cell membranes in order to investigate the effects of pH perturbations in intracellular compartments on transport and processing of viral proteins and on the incorporation of viral proteins into mature virions (31). In previous studies, other investigators have shown monensin to have multiple effects on protein transport in both the endocytic and exocytic pathways (31). Transport and processing of virus glycoproteins is blocked or altered, the block usually occurring in the medial region of the Golgi vesicles (11). Protein molecules that pass this block are often improperly glycosylated and usually contain a mixture of unprocessed mannose-rich oligosaccharides (11). Monensin also can affect steps in protein processing that occur after passing through the Golgi in post-Golgi vesicles. For example, cleavage of the insulin receptor precursor protein is blocked in monensin-treated cells. This cleavage normally occurs in post-Golgi transport vesicles (20).

Monensin has been shown to have a variety of related effects on the transport of the glycoproteins of Sindbis virus and Semliki Forest virus. It inhibits virus budding from the cell surface (13, 15). Transport of envelope glycoprotein precursors is totally or partially blocked in the Golgi vesicles in the presence of monensin (11, 13). In cells infected with Semliki Forest virus or Sindbis virus, aberrant budding of viruslike structures into the lumen of intracellular vesicles occurs. Aberrant glycosylation of Semliki Forest virus and Sindbis virus glycoproteins also occurs. Some high-mannose

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glycosylations are not trimmed, while others are processed to complex oligosaccharides with an abnormally low sialic acid content (11, 13).

MATERIALS AND METHODS

Cells and virus. Baby hamster kidney (BHK-K) cells were grown as described previously (21) in Eagle minimum essential medium (8) supplemented with 5% fetal calf serum, 5% tryptose phosphate broth, and 2 mM glutamine. The heat-resistant strain of Sindbis virus was propagated in BHK-K cells as described previously (21). Virus was routinely purified by isopycnic gradient centrifugation in potassium tartrate as described previously (9).

Polyacrylamide gel electrophoresis. Electrophoresis of radiolabeled proteins was carried out under denaturing conditions in 11.8% acrylamide as described previously (25). Sample buffer was prepared as described previously (25). Fluorography of the gels was performed by the method of Bonner and Laskey (1).

Radiolabations. Lactoperoxidase-catalyzed iodinations were performed as previously described (29), except that iodination of cells was performed on intact monolayers in which the medium had been removed and replaced with 1 ml of phosphate-buffered saline (PBS). Iodinations were terminated by the addition of a 40-fold excess of nonradioactive iodine. The PBS was then removed, the monolayers were lysed in 0.5% Nonidet P-40, and virus proteins were immunoprecipitated with anti-Sindbis virus serum.

Metabolic labeling of cells and virus. Metabolic labeling was performed with [35S]methionine or with a mixture of [35S]methionine and [35S]cysteine, at a concentration of 20 to 50 μCi/ml. Monolayers were pretreated with 4 μg of actinomycin D per ml for 90 min before infection, and this concentration of actinomycin D was maintained in the medium throughout the labeling process. Monolayers were starved for 1 h with methionine-deficient or methionine- and-cysteine-deficient medium before labeling and maintained in methionine-deficient medium during the pulse. The presence of monensin during the pulse did not affect incorporation of label at the concentrations of drug used in this study. Labeling was terminated by washing the monolayers with medium containing 10 times the normal concentration of methionine and 50 μg of cycloheximide per ml.

Immunoprecipitations of radiolabeled virus proteins were then performed by using anti-Sindbis virus rabbit antiserum and staphylococcal protein A conjugated to Sepharose beads. Monolayers were lysed in 1 ml of a 0.5% Nonidet P-40 solution (Sigma Chemical Co.) containing 0.02 M Tris, 0.05 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM N-tosyl-L-phenylalanine chloromethyl ketone (Sigma), and 0.2 mM N-acetyl-L-lysine chloromethyl ketone (Sigma) (16). The lysate was then treated with 5 μl of rabbit anti-Sindbis virus serum and 400 μl of 10% protein A-Sepharose beads (Sigma) suspended in lysis buffer and allowed to sit overnight. The bead-antibody complexes were then washed three times with lysis buffer and suspended in sample buffer.

Immunological assays. Monolayers in 24-well plates were infected with Sindbis virus at a multiplicity of infection of 50. At 6 h postinfection, wells to be assayed were washed extensively with PBS. PBS (190 μl) was added to each well, along with 10 μl of rabbit anti-Sindbis virus antiserum. Monolayers were then put on ice for 1 h and afterward extensively washed with PBS. Iodinated (1 μCi) staphylococcal protein A (Dupont, NEN Research products; 37-μCi 125I per mg of protein A) was added to each well, which was then washed twice with PBS to remove unbound protein A. Noninfected-cell monolayers were similarly treated to determine nonspecific binding. Monolayers were lysed with 2% sodium dodecyl sulfate in PBS, and radioactivity was quantitated in a Tri-Carb model 3003 scintillation counter (Packard Instrument Co., Inc.).

Drugs and chemicals. A 1 mM monensin stock solution was made by dissolving monensin in ethanol and stored at −20°C. Fresh stock solutions were made up at intervals. Samples from stock solutions were added to medium in tubes and vortexed. Medium containing the proper concentration of monensin was then added to the monolayers. Monensin was purchased from Sigma Chemical Co.

RESULTS

Effect of monensin on virus production. Cells were treated with various concentrations of monensin at 30 min postinfection to circumvent any effect this drug may have on the early events in virus infection (7, 12). Virus production 6 h postinfection was determined by plaque assay, and the dose-response curves presented in Fig. 1A were obtained. A dose of 1 μM monensin resulted in reduction of virus titer to 16% of control value, while a dose of 5 μM resulted in complete inhibition of virus production. These results are similar to those obtained by Johnson and Schlesinger (13).

Effect of monensin on transport of virus protein to the cell surface. An immunological assay similar to that described by Erwin and Brown (10) or by Mann et al. (19) was used to quantitate virus protein on the surface of monensin-treated, virus-infected cells (Fig. 1B). Anti-Sindbis virus antibody bodies were bound to infected cells at 6 h after infection, and antibody binding was quantitated by the binding of iodinated staphylococcal protein A. Monensin (1 μM) added 30 min postinfection reduced virus protein transport to the cell surface by approximately 50%. Doses of 5 μM or higher reduced transport to levels not detectable above background. This suggests that the drop in virus production seen at a dose of 1 μM monensin (to 16% of control value) is not entirely the result of failure of glycoprotein to transport to the cell surface (50% of control [Table 1]).

Effect of monensin on the cleavage of pE2. Monensin has been shown to inhibit the cleavage of pE2 to E2 in Sindbis virus-infected cells (13). We have repeated these studies to establish a correlation between the concentration of drug reducing transport to the cell surface and the concentration required to inhibit the cleavage of pE2 to E2. To quantitate this effect, we pulse-labeled methionine-starved cells for 7.5 min at 6 h postinfection with [35S]methionine and chased for 45 min in the presence of cycloheximide and monensin. Monolayers were lysed in Nonidet P-40 and immunoprecipitated envelope proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and quantitated by integrating the corresponding peaks in densitometric scans of the autoradiogram (see Materials and Methods). We found (Fig. 1C) that cleavage was inhibited by doses of monensin insufficient to completely block virus production. A dose of 0.1 μM monensin or greater caused complete inhibition of cleavage of pE2 to E2, as assayed after a 45-min chase. It is important to note that a dose of 1 μM was sufficient to almost totally block cleavage of pE2 to E2, yet it reduced virus protein on the cell surface by only around 50% of control value (Table 1). Since processing of pE2 to E2 could be delayed in drug-treated cells, we treated cells with 1 μM monensin as described above and extended the
TABLE 1. Effects of various doses of monensin on Sindbis virus protein processing

<table>
<thead>
<tr>
<th>Concentration (μM) of monensin</th>
<th>Virus production</th>
<th>Protein on cell surface</th>
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<tbody>
<tr>
<td></td>
<td>% Control</td>
<td>PE2:E2</td>
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<tr>
<td>0.01</td>
<td>57</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1</td>
<td>23</td>
<td>2.4</td>
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<tr>
<td>1.0</td>
<td>16</td>
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</tr>
<tr>
<td>5.0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10.0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
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<sup>a</sup> The value of antibody binding of the uninfected control was subtracted from all values in the column showing virus protein measured on the cell surface before conversion of these numbers to percentages.

<sup>b</sup> ND, Not determined.

-chase time to 2 h. A small fraction of pE2 (4.1% of the total labeled) was converted to E2 at this time point (data not shown).

**Surface protein iodination.** The preceding results show that a dose of 1 μM monensin could greatly reduce the cleavage of pE2 to E2, as assayed in a 45-min chase, without completely preventing transport of virus-specific protein to the cell surface. We therefore chose this concentration of monensin to lower the pH of the post-Golgi transport vesicles and to determine the effects of this alteration on the virus envelope proteins.

Lactoperoxidase-mediated iodination was employed to examine the conformation of pE2 or E2 on the surface of cells infected with Sindbis virus and treated with monensin (see Materials and Methods). Treatment with 1 μM monensin at 30 min postinfection resulted not only in a reduction in the amount of E2 detectable on the cell surface at 6 h postinfection but also (and more interestingly) in the appearance of pE2 in a conformation detectable by iodination (Fig. 2). The amount of pE2 detectable on the cell surface relative to E2 was increased if the dose of monensin was increased to 10 μM. The Sindbis virus capsid protein was not iodinated in any of these experiments. This protein is inaccessible to the enzyme lactoperoxidase by virtue of its cytoplasmic location, and failure to iodinate this protein demonstrates that the labeling of pE2 in this experiment does not result from increased permeability or lysis of cells by monensin (27, 29).

pE2 was also detected by iodination on the surface of cells treated with various doses of the lysosomotropic weak bases chloroquine (Fig. 2) and ammonium chloride (data not shown), indicating that this effect was not specific to monensin. CHO cell lines defective in the acidification of endosomal and post-Golgi compartments have been shown by others to express iodinatable pE2 on the cell surface when infected with Sindbis virus (23), giving further evidence that raising the pH of the post-Golgi compartment by itself is sufficient to cause the appearance of pE2 on the cell surface in an iodinatable conformation.

**FIG. 1.** (A) Effects of monensin on virus titer measured by plaque assay 6 h postinfection and graphed as percent control value (no monensin). (B) Effect of monensin on quantity of virus protein in the plasma membrane, was measured by binding of anti-Sindbis virus antibody to monolayers and by quantitation of this antibody by...
Maturation of Sindbis virus in monensin-treated cells. Cells treated with moderate concentrations of monensin were found to produce a limited amount of infectious virus (Table 1). We examined the protein composition of virus purified from the medium of monensin-treated infected cells to determine if this virus was produced from the small quantity of E2 which was produced in the presence of drug. This would indicate that the pE2 in an iodinatable conformation on the cell surface was nonfunctional. Virus was purified from the medium by isopycnic centrifugation in 20-to-35% potassium tartrate gradients. The virus produced sedimented normally in a 7.5-to-30% sucrose gradient and showed normal morphology when negatively stained with uranyl formate and examined by electron microscopy (data not shown). Pelleted virus was dissolved in sample buffer, and virus proteins resolved by electrophoresis as described in Materials and Methods. Virus produced from cells treated with 1 μM monensin contained significant amounts of pE2. Densitometer tracings of the fluorogram of the gel, normalized for the number of methionines in each protein (30) showed that pE2 exceeded E2 by a ratio of approximately three to one. The stoichiometric relations of the envelope proteins revealed that the relationship of the amounts of pE2 and E2 equaling that of E1 remained unchanged, suggesting that the virus population produced from monensin-treated cells contains 75% of one of its two envelope glycoproteins as the precursor pE2. It seems likely that, in the process of virion assembly occurring at the plasma membrane, pE2 was paired to E1 and substituted for E2, which in normal virus is present in equimolar amounts to E1.

Infectivity of pE2-containing virus. That Sindbis virus can be produced containing a large amount of pE2 shows only that the conversion of pE2 to E2 is not strictly required for budding. The reduced amount of virus produced from monensin-treated cells may result from the inhibitory effects this drug has on the transport of proteins to the plasma membrane, rather than an inhibition of pE2 processing (Table 1). To compare the infectivity of this virus to virus from untreated cells, we quantitated the protein present in purified virus from untreated monolayers and from monolayers treated with 1 μM monensin. Protein was quantitated by the Bio-Rad assay, and the infectivity from roughly equivalent samples was compared by plaque assay (Table 2). If the presence of pE2 in virions rendered the particles noninfectious or if the virus produced in the presence of monensin consisted of two populations, one noninfectious containing pE2-E1 and the other infectious containing E2-E1, one would have expected many fewer infectious virions per microgram of protein in the virus produced in the presence of monensin. PE2-containing virus populations were found to be as infectious as virus containing only E2.

DISCUSSION

The cleavage of the E2 precursor pE2 to E2 has long been believed to be an event required for virus maturation in the Alphaviridae. This cleavage was at one time believed to be coupled to virus budding (2, 14, 29). This conclusion was

<table>
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<th>Virus</th>
<th>Infectivity of virus containing pE2</th>
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<tr>
<td></td>
<td>Bio-Rad (μg/ml)</td>
</tr>
<tr>
<td>Control</td>
<td>23.7</td>
</tr>
<tr>
<td>Monensin-treated</td>
<td>30.0</td>
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* Virus was purified by isopycnic centrifugation in potassium tartrate. A sample was then removed for plaque assay. The protein concentration in the remainder was determined by the Bio-Rad assay.
based on several lines of evidence. Studies in which anti-E1 or anti-E2 antibodies block the cleavage of pE2 to E2 as well as budding originally suggested a coupling between budding and cleavage. Later work led to the abandonment of the proposed obligatory link between budding and cleavage but strengthened the idea that the cleavage is at least an obligatory precondition for budding (13, 22). This idea was reinforced further by the phenotype of the mutant ts20, which has a lesion in E2 and fails to cleave pE2 to E2 (3, 25). Protein is transported to the cell surface (29), and can bind capsid (3), demonstrating that pE2 and not merely E1 is present at the cell surface (25).

The data presented above show that a drug which affects the ability of cells to acidify internal vesicles including those vesicles responsible for the transport of proteins destined for the plasma membrane can, when applied at low concentrations, allow the transport of glycoprotein to the plasma membrane in an atypical conformation. Sindbis virus glycoprotein pE2 is detected at the cell surface in a conformation allowing its iodination by the enzyme lactoperoxidase. This atypical pE2 is incorporated into mature infectious virus in quantities showing that it can at least partially substitute for E2. This does not rule out the possibility that some E2 is required to produce a viable virion in our strain of Sindbis virus. However, in a recent paper, Russell et al. (24) have shown that, in at least one strain of Sindbis virus (S.A.AR86), mutants can be produced in which the cleavage of pE2 is blocked totally, yet infectious virus is efficiently produced.

These observations suggest that the pE2 or E2 protein undergoes dramatic conformational changes during transport to the cell surface. Figure 3, in schematic form, one possible explanation for how these changes may occur. In normal cells, pE2 is initially in a conformation exposing tyrosine residues to the enzyme lactoperoxidase. It is transported to the cell surface in vesicles that are progressively acidified by a proton pump associated with their membrane (20, 31). As pE2 is exposed to acid conditions, it is assumed to assume a conformation in which its tyrosine residues are protected. While in this conformation, pE2 may be efficiently cleaved to E2, which then undergoes a conformational change, exposing its tyrosine residues to enzyme-mediated iodination. This conformational change may result directly from the cleavage event or from a combination of cleavage and exposure to neutral pH conditions as the protein is exposed at the cell surface. We have found that the mature E2 found in virions undergoes a dramatic change in conformation upon exposure to acid conditions. This conformation renders the protein sensitive to trypsin (9) and its tyrosine residues insensitive to enzymatic radioiodination (J. Edwards and D. T. Brown, unpublished observations). This conformation may be similar to the conformation induced in the intracellular pE2 by acid conditions during transport.

In the presence of low concentrations of monensin, pE2 is transported to the cell surface in vesicles that fail to acidify and arrives in its iodinatable conformation. It is not cleaved yet can be incorporated into virions in place of E2.

These results suggest that processing of Sindbis virus glycoprotein pE2 to E2 is not absolutely required for virus maturation. This observation further suggests that failure to produce virus with mutant ts20 (at nonpermissive temperature) is not simply the result of failure to cleave pE2. The alteration in the primary structure of ts20 pE2 may result in its improper folding on exposure to acid environments during transport to the plasma membrane. The observations of Smith and Brown indicate that this pE2 is not iodinatable (29).

The data presented above show that Sindbis virus containing pE2 substituting for E2 is as infectious as normal virus (Table 2). In a recent paper, Russell and coworkers (24) have reached similar conclusions by producing a mutant virus (S.A.AR86 S12), totally incapable of cleaving pE2 to E2, which yields viable progeny. In preliminary studies, we have found that virus production by S.A.AR86 S12 in BHK 21 cells is arrested at a concentration of monensin lower than is required to produce a similar arrest of heat-resistant Sindbis virus production (data not shown). This observation implies that the inhibition of virus production by monensin is not exclusively due to its ability to prevent cleavage of pE2.

If the cleavage of pE2 to E2 and E3 is not essential for production of infectious virus, what is its function? Alphaviruses evolutionarily preserve this cleavage event, suggesting that it gives a strong selective advantage at some point in virus assembly or in the virus life cycle in nature. It is possible that this cleavage event is, for some reason, absolutely required in mosquito cells, which form an indispensable part of the virus life cycle in nature. Alternatively, it may alter pathogenesis in animals, improve virus stability, or confer some more subtle advantage, such as protection from immune surveillance. Sindbis virions containing pE2 could be altered or defective for a number of characteristics, including stability at high temperatures, ability to fuse cells at low pH, or ability to grow in mosquito cells. These possibilities are, at present, under investigation.

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LITERATURE CITED
