Brefeldin A and monensin arrest cell surface expression of membrane glycoproteins and release of rubella virus

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The maturation of rubella virus (RV) glycoproteins E2 and E1 was examined by using brefeldin A (BFA) and monensin. BFA, which induces the rapid redistribution of Golgi enzymes residing in the Golgi complex into the endoplasmic reticulum (ER), was used to locate the intracellular site for the modification of carbohydrate side-chains on RV E1 and E2 proteins. The monovalent ionophore monensin, which inhibits intracellular transport of proteins through the ER-Golgi complex, was used to block the transport of E1 and E2 glycoproteins through the Golgi complex. BFA and monensin effectively blocked the cell surface expression of RV E2 and E1 proteins, secretion of an anchor-free form of E2 and budding of RV from the plasma membrane. For O-linked glycosylation, addition of N-acetylgalactosamine and galactose to E2 protein was found to take place in the medial to the trans Golgi. A dramatic change in the intracellular distribution of RV structural proteins was observed when transfected COS cells were treated with BFA or monensin, although the proteolytic processing of RV structural protein precursor was not affected. In the presence of BFA or monensin, virus release from infected Vero cells was only 0.1% of the intracellular virus, and the intracellular virus titre decreased as well. Our results suggest that O-linked glycosylation on the E2 protein occurred in the post-ER region and the transport of RV structural proteins to the Golgi complex and post-Golgi compartment may be a rate-limiting step in RV assembly and budding.

Introduction

Rubella virus (RV) is a small enveloped positive-stranded RNA virus in the Togaviridae family (Matthews, 1982). In the RV virion, the icosahedral nucleocapsid is composed of a single copy of the 40S genomic RNA and multiple copies of the capsid protein (C, 33 kDa), surrounded by a lipid bilayer, into which two viral glycoproteins E1 (57 kDa) and E2 (42-47 kDa) are embedded (Vaheri & Hovi, 1972; Oker-Blom et al., 1983). In addition to the 40S genomic RNA, RV-infected cells contain a 24S subgenomic RNA derived from the 3' end of the 40S genomic RNA (Oker-Blom et al., 1984). A polyprotein precursor (p110) is translated from the 24S subgenomic RNA and is subsequently processed to produce three structural proteins (Oker-Blom et al., 1984).

The processing of RV structural proteins involves a series of co-translational and post-translational modifications. Expression of RV structural proteins in vitro and in vivo from cDNA constructs demonstrates that cleavage of the polyprotein precursor is carried out by cellular signal peptidase(s) during translocation through the endoplasmic reticulum (ER) (McDonald et al., 1991; Qiu et al., 1994a). N-Linked glycosylation of E1 and E2 is initiated in the ER and is essential for efficient transport and processing of E1 and E2 as they traverse the secretory pathway (Hobman et al., 1990; Qiu et al., 1992). High-mannose, hybrid-type and complex-type N-linked glycans have been found in both E1 and E2 proteins from RV virions (Sanchez & Frey, 1991). O-Linked oligosaccharides are observed only in E2 (Sanchez & Frey, 1991; Lundström et al., 1991). Formation of E1–E2 heterodimers in the ER (Baron & Forsell, 1991) facilitates the transport of E2 (Hobman et al., 1990) and E1 (Baron et al., 1992; Hobman et al., 1993) to the Golgi complex.

Little is known about the mechanism of RV assembly and budding. In BHK-21 cells, maturation occurs primarily in the Golgi or vacuoles and to a lesser extent at the plasma membrane, whereas in Vero cells, virions bud mostly from the plasma membrane (Payment et al., 1975; Bardeletti et al., 1979; von Bonsdorff & Vaheri, 1969). In RV-infected cells, the majority of E1 and E2 proteins accumulated in the Golgi-like region with a limited amount located at the cell surface (Hobman et al., 1990). The budding process is believed to be driven...
by the interaction of spike proteins and nucleocapsid. Thus the site of virus maturation may be determined, at least in part, by the localization of these proteins. The abundance of RV glycoproteins in the Golgi complex may be crucial to the ability of the virus to bud from intracellular membranes (Bardeletti et al., 1979).

Brefeldin A (BFA) is a fungal metabolite that disrupts intracellular membrane traffic at the ER—Golgi junction (Misumi et al., 1986). Monensin is a well-characterized metabolite of Streptomyces cinnamoneus that binds Na*, K* and proteins (Pressman, 1976). BFA and monensin are two compounds that have been used effectively to inhibit the transport and secretion of a number of cellular and viral proteins in a variety of cell lines (Misumi et al., 1986; Fujiwara et al., 1988; reviewed by Mollenhauer et al., 1990). In general, for viruses (e.g. vesicular stomatitis virus and Sindbis virus) that bud at the plasma membrane, BFA (Oda et al., 1990) and monensin (Johnson & Schlesinger, 1980) inhibit virus assembly and release as the result of a complete blockade of cell surface expression of viral envelope glycoproteins. By contrast, for Punta Toro virus, which assembles at the Golgi complex, BFA blocks virus release from cells but does not interfere with the intracellular assembly of infectious virions, while monensin decreases the yield of both cell-associated and released virus as the concentration is increased (Chen et al., 1991).

In RV virions, E2 glycoprotein exists as multiple protein species with molecular mass values ranging from 42 to 47 kDa (Oker-Blom et al., 1983). It has been suggested that this is due to heterogeneous glycosylation. In order to investigate the possibility that the heterogeneity of N-linked glycosylation and the presence of O-linked glycans on E2 protein may be important in governing the assembly and budding of RV, we have used BFA and monensin to study the effect of post-translational processing of RV glycoproteins on virion maturation and the secretion of RV E2 proteins in COS cells. In this study we showed that RV glycoproteins synthesized in the presence of BFA were fully processed by medial and trans Golgi carbohydrate-processing enzymes, whereas monensin inhibited the transport of RV glycoproteins from the medial to the trans Golgi. The effect of BFA and monensin on virus infectivity paralleled that of the disruption of distribution of E1 and E2 in the Golgi complex, suggesting the possibility that a stable association of E1 and E2 in the Golgi complex may be essential for efficient RV assembly and budding at the plasma membranes.

**Methods**

*Chemicals and antibodies.* Brefeldin A was purchased from Boehringer Mannheim and kept as a 1 mg/ml stock in methanol at −20 °C. Monensin was from Sigma and kept as a 1 mM stock in ethanol at −20 °C. Human polyclonal anti-rubella serum was provided by Dr A. Tingle (Department of Pediatrics, B.C.'s Children's Hospital, Canada). Mouse monoclonal antibodies against RV E1 were generated in the laboratory of S.G. Mouse monoclonal antibodies against RV E2 and capsid protein were generously provided by Dr J. Safford (Abbott Laboratories). Rabbit polyclonal antibodies against Golgi protein fraction were prepared in the laboratory of F.T.

**Cells and virus.** COS, Vero and RK cells were obtained from the ATCC. COS cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 5% fetal calf serum (Gibco). Monolayer cultures of Vero cells were grown in minimal Eagle's medium (MEM) supplemented with 5% fetal bovine serum. RK cells were grown in M199 medium with 5% fetal calf serum. Rubella virus M33 strain was utilized throughout.

**Plasmids.** The construction of pCMV5-24S (encoding all three structural proteins) and pCMV5-E2 (encoding E2 protein) has been described previously (Hobman et al., 1990). All cDNAs were subcloned between the EcoRI and HindIII sites of the expression vector pCMV5 (Andersson et al., 1989). pCMV5-E2A3' was constructed by deleting 68 amino acids at the C terminus of E2 including the putative transmembrane domain of E2 (Hobman et al., 1994).

**Labelling of cells and analysis of viral proteins.** (i) COS cells. COS cells were transfected with recombinant plasmids using DEAE-dextran as described by Adams & Rose (1985) with modifications (Hobman & Gillam, 1989). Labelling of cells and immunoprecipitation of RV proteins were performed according to Hobman & Gillam (1989). Briefly, 48 h post-transfection, transfected cells were labelled with 100 μCi [35S]methionine (Du Pont) for 30 min. Cells were incubated with a chase medium containing excess unlabelled methionine for the indicated periods of time. Cells were lysed and RV-specific proteins were recovered by immunoprecipitation using human anti-RV serum, subjected to SDS—PAGE and fluorography (Hobman & Gillam, 1989). For some samples, immunoprecipitation was performed on the culture medium to recover secreted E2 in the medium. Digestion of immunoprecipitated proteins by endoglycosidase H (endo H), endo-glycosidase F/N-glycosidase F (endo F/PNGase F) and O-glycosidase (all from Boehringer Mannheim) was performed as described (Hobman & Gillam, 1989; Lundström et al., 1991).

(ii) Vero cells. Monolayers of Vero cells were infected with RV M33 at an m.o.i. of 5–10 as described (Clarke et al., 1987). Twenty-four hours post-infection (h.p.i.), infected cells were incubated with methionine-deficient medium for 30 min and labelled with 100 μCi [35S]methionine for 1 h. Cells were washed with and incubated in MEM with 2.5% fetal calf serum for the indicated periods of time. Samples were collected and RV particles were precipitated with 10% polyethylene glycol (mol. mass 8000). The virus pellets were resuspended in RIPA buffer (1% Triton X-100; 10 mM-EDTA; 50 mM-Tris-HCl, pH 7.5; 1% sodium deoxycholate; 0.15 mM-NaCl; 0.1% SDS) and RV-specific proteins were immunoprecipitated with human anti-RV serum and subjected to SDS—PAGE and autoradiography. Indirect immunofluorescence. Transfected COS cells were first treated with mouse monoclonal antibodies against individual RV structural proteins, or human anti-RV serum, followed by incubation with fluorescein (FITC)-conjugated goat anti-mouse or anti-human IgGs (Hobman & Gillam, 1989). A tetramethylrhodamine isothiocyanate (TRICT)-conjugated rabbit anti-Golgi protein serum was used to visualize Golgi structure.

**One-step multiplication assay.** Monolayers of Vero cells in 24-well plates were infected with RV at an m.o.i. of 10 at 37 °C for 2 h. The infected cells were washed with and incubated in MEM with 2.5% fetal bovine serum. BFA (6 μg/ml) or monensin (5 μM) were added to the medium at 8 h p.i. and re-supplemented at 12 h intervals. At 24, 36, 48 and 60 h p.i., medium samples (1 ml/well) were collected.
associated viruses were recovered by freezing and thawing the cells and were dissolved in 1 ml phosphate-buffered saline. The infectivity of intracellular and extracellular viruses was determined using an immunochemical focus assay (Fukuda et al., 1987). Briefly, monolayers of RK cells in a 96-well plate were infected with RV in serial dilutions. Infected cells were fixed with formaldehyde at 72 h p.i. and incubated with human anti-RV serum. Cells were then incubated with peroxidase-conjugated rabbit anti-human IgG. Plaques were visualized after applying peroxidase substrate and titres of virus were expressed as p.f.u./ml.

## Results

### Effect of BFA and monensin on RV E2 glycan processing

RV E2 protein is heavily glycosylated. Its glycosylation processing in RV virions is heterogeneous and differently glycosylated species may be present in E2 (Bowden & Westaway, 1985). It has been shown that E2 from the RV M33 strain contains three large complex-type N-linked and several smaller O-linked carbohydrates (Lundström et al., 1991). Pulse-chase experiments of E2 expressed from cDNA have demonstrated that E2 glycoprotein is synthesized predominantly as a 37 kDa endo H-sensitive species at the end of 30 min pulse-labelling and is further processed to a 42 kDa protein containing partially endo H-resistant sugar moieties during the chase period (Hobman et al., 1990; Qiu et al., 1992). The pattern of glycan processing and heterogeneity of carbohydrates on E2 make it a useful system to study the effect of BFA and monensin on these events, as well as to define the subcellular sites for specific carbohydrate processing.

To determine the appropriate concentrations of BFA and monensin for the study, COS cells were transfected with pCMV5-E2 and labelled with [35S]methionine at 40 h post-transfection. Either BFA or monensin at various concentrations was added to the culture medium 2 h prior to the labelling and was present in the medium throughout the experiment. Interestingly, BFA seemed to enhance the level of E2 protein expression (Fig. 1 a), the mechanism of which is not understood. However, no significant difference in the glycan processing of RV E2 was found in cells treated with BFA in the range from 1 μg/ml to 12 μg/ml (Fig. 1 a). Thus 6 μg/ml of BFA was chosen to use in the subsequent analysis. In contrast, monensin at higher concentration (25 μM) inhibited E2 synthesis (Fig. 1 b) and 5 μM-monensin (d) were present. Cells were lysed with RIPA buffer after labelling (a, b) or after the chase (c, d). E2 proteins were immunoprecipitated with human anti-RV serum, then subjected to SDS-PAGE and fluorography. Half of each immunoprecipitated sample was digested with endo H (+) for 8 h at 37 °C before separating on SDS-PAGE. Molecular mass markers (kDa) are shown on the left. E2 protein bands containing endo H-sensitive (s) and -resistant (r) sugar moieties are marked. The novel 34 kDa E2 protein species is indicated by an asterisk (*).

![Fig. 1. Effect of BFA or monensin on processing of E2. Transfected cells were pulse-labelled with 100 μCi [35S]methionine for 30 min and incubated with excess methionine for the indicated time in chase experiments (c, d). BFA (in μg) or monensin (in μM) were added to the medium at the concentrations shown in (a) and (b). In chase experiments, 6 μg/ml BFA (c) or 5 μM-monensin (d) were present. Cells were lysed with RIPA buffer after labelling (a, b) or after the chase (c, d). E2 proteins were immunoprecipitated with human anti-RV serum, then subjected to SDS-PAGE and fluorography. Half of each immunoprecipitated sample was digested with endo H (+) for 8 h at 37 °C before separating on SDS-PAGE. Molecular mass markers (kDa) are shown on the left. E2 protein bands containing endo H-sensitive (s) and -resistant (r) sugar moieties are marked. The novel 34 kDa E2 protein species is indicated by an asterisk (*).](image-url)
**Fig. 2.** Glycosidase digestion of E2 from BFA- or monensin-treated cells. Transfected cells were labelled with [³⁵S]methionine for 30 min and incubated with medium containing excess methionine for 4 h before lysis with RIPA buffer. E2 proteins were immunoprecipitated with human anti-RV serum, digested with glycosidase for at least 8 h at 37 °C and subjected to SDS–PAGE and fluorography. BFA (6 μg/ml), monensin (5 μM) and tunicamycin (3 μg/ml) were present in the medium where applicable. Digestion with endo F/PNGase F (N-gly), neuraminidase (Neu) and O-glycosidase (O-gly) are indicated. Molecular mass markers (kDa) are included for reference. (a) Untreated cells; (b) cells treated with BFA; (c) cells treated with monensin. Tunicamycin (Tm) was present in the medium of some transfected cells as indicated.

Fig. 3. Effect of BFA or monensin on processing and secretion of an anchor-free form of E2. COS cells were transfected with recombinant plasmid containing cDNA encoding a secreted form of E2 and labelled with 100 μCi [³⁵S]methionine for 30 min at 40 h post-transfection. After incubation with excess methionine for 4 h, medium samples were collected and cells were lysed. E2 proteins were immunoprecipitated with human anti-RV serum from medium as well as cell lysates, separated on SDS–PAGE and visualized by autoradiography. Half of each sample was digested with endo H (+). Molecular mass markers (kDa) are shown on the left. Control, E2 from untreated cells; BFA, E2 from BFA-treated cells; Mon, E2 from monensin-treated cells.

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It has been shown that E2 contains O-linked oligosaccharides (Lundström et al., 1991; Sanchez & Frey, 1991). It is of interest to analyse the O-linked glycosylation of E2 when cells were treated with BFA or monensin in the presence and absence of tunicamycin. Transfected COS cells were labelled with [³⁵S]methionine and chased for 4 h. In control cells (untreated), digestion with endo F/PNGase F reduced the molecular masses of two E2 species from 42 kDa and 37 kDa to 36 kDa and 31 kDa, respectively (Fig. 2a), similar to changes observed after endo H digestion (Fig. 1c). After incubation with neuraminidase, the 37 kDa E2 remained unchanged, whereas the 42 kDa species was reduced to 40 kDa (Fig. 2a). This 40 kDa species was diminished after further digestion with O-glycosidase (Fig. 2a). Deglycosylation of E2 with a combination of endo F/PNGase F, neuraminidase and O-glycosidase resulted in a major unglycosylated 31 kDa E2 species (Qiu et al., 1992) and a minor 34 kDa species that may be due to the incomplete removal of O-linked oligosaccharide (Fig. 2a). Treatment with tunicamycin abolished the N-linked glycosylation, as well as the addition of O-linked oligosaccharide on E2, as only a 30 kDa unglycosylated E2 species was observed (Fig. 2a). The cause of the 1 kDa difference in molecular mass between E2 deglycosylated by glycosidase digestion and unglycosylated E2 (by tunicamycin treatment) is not known.

In BFA-treated cells, the single 42 kDa E2 species was reduced to 37 kDa by endo F/PNGase F digestion; to 39 kDa by neuraminidase digestion; or to 37 kDa by further incubation with O-glycosidase (Fig. 2b). Digestion with a combination of endo F/PNGase F, neuraminidase and O-glycosidase resulted in a single protein species of 31 kDa (Fig. 2b). In cells treated with both tunicamycin and BFA, two protein species with apparent molecular masses of 31 kDa and 35 kDa were observed (Fig. 2b). Since N-linked glycosylation was
inhibited by tunicamycin, the 35 kDa species must contain only O-linked glycans. Indeed, digestion with neuraminidase reduced its molecular mass to 33 kDa while further incubation with O-glycosidase gave rise to a single protein species of 30 kDa (Fig. 2b). In monensin-treated cells, E2 ran as a smeary band with molecular mass ranging from 37 kDa to 42 kDa. A distinctive protein species with a molecular mass of 34 kDa was observed in endo F/PNGase F-digested samples as well as in samples digested with a combination of endo F/PNGase F, neuraminidase and O-glycosidase (Fig. 2c). This 34 kDa protein material could represent intermediates of O-linked glycans that accumulated in monensin-treated cells and which were not accessible to O-glycosidase. In cells treated with monensin and tunicamycin, no O-linked glycosylation was observed (Fig. 2c).

Taken together, these results suggest that E2 is first synthesized in the ER as a 37 kDa protein species containing only N-linked glycans, and as it reaches the Golgi complex, O-linked glycosylation takes place to increase the molecular mass of E2 from 37 kDa to 42 kDa. BFA treatment caused a redistribution of Golgi enzymes into the ER and resulted in a rapid addition of O-linked sugars on E2 in the ER, even when N-linked glycosylation was inhibited by tunicamycin. In monensin-treated cells, the O-glycosylation of E2 was not abolished, but it was processed aberrantly.

Processing and secretion of an anchor-free form of E2

To study further the transport of E2 along the secretory pathway in BFA- or monensin-treated cells, a cDNA construct encoding an anchor-free form of E2 was transfected into COS cells and the expressed E2 protein was analysed. In control cells, soluble E2 was secreted from the transfected cells into the culture medium at a ratio of 10–17% of total E2 protein (data not shown). The secreted E2 was found to be resistant to endo H digestion (Fig. 3), indicating that it had been modified by glycan processing enzymes as it traversed the secretory pathway. The majority of intracellular E2 was endo H-sensitive (Fig. 3). In contrast, although the intracellular form of soluble E2 in BFA-treated cells was partially or completely resistant to endo H digestion (Fig. 3), no secreted E2 was detected (Fig. 3). Monensin completely inhibited the secretion of E2 from transfected cells, whereas intracellular E2 exhibited no obvious difference from that of control cells (Fig. 3). Taken together, it is evident that BFA and monensin perturb E2 transport in the secretory pathway and completely block the movement of E2 to the cell surface.

Effect of BFA and monensin on proteolytic processing of RV structural protein precursor

To study the effect of BFA and monensin on the processing of RV polyprotein precursor, a recombinant plasmid containing RV cDNA encoding all three structural proteins (pCMV5-24S) was used to transfect COS cells. The expression of the polyprotein precursor and the proteolytic processing of the precursor were analysed by pulse-chase experiments. At the end of a 30 min labelling, the majority of RV structural proteins were present as individual polypeptides, although some E1/E2 uncleaved precursor proteins with higher molecular masses were also observed (Fig. 4, pulse). These minor polyprotein species were not seen after a 3 h chase period (Fig. 4, chase). There was no significant difference in the amount of cleavage products for RV-specific proteins between the control cells and BFA- or monensin-treated cells, indicating that BFA and monensin did not directly affect the proteolytic processing of RV structural protein precursor. This is
consistent with our previous finding that the cleavage of polyprotein precursor is conducted by cellular signal peptidases (McDonald et al., 1991; Qiu et al., 1994a), and suggests that it is an ER-specific event not interrupted by the influx of resident Golgi proteins upon BFA treatment or by an impaired Golgi upon monensin treatment. We have shown that RV structural proteins are assembled into virus-like particles in the absence of genomic RNA and released from the cells into the medium (Qiu et al., 1994b). This is probably the reason that there was a slight decrease in the amount of RV structural proteins in control cells after a 3 h chase period (Fig. 4, chase).

**Subcellular distribution of RV structural proteins in BFA- or monensin-treated cells**

Indirect immunofluorescence was used to localize the RV structural proteins in cells transfected with pCMV5-24S. In control cells, E2 was found to be concentrated in the juxtanuclear region co-localized with a Golgi marker (Fig. 5a, b), while E1 was found in the peri- and juxtanuclear region corresponding to the ER and Golgi structure (Fig. 5c, d). The capsid protein was distributed throughout the cytoplasm (Fig. 5e). A limited amount of E2 and E1 was detected at the cell surface (Fig. 5f). In BFA-treated cells, E2 and E1 displayed a predominant perinuclear staining pattern which co-localized with a Golgi marker (Fig. 5g–k). Combined with the results from pulse-chase analysis, it appeared that in transfected COS cells, BFA treatment caused Golgi-resident proteins to cycle back into the ER (as shown by the perinuclear staining with the Golgi marker) and blocked the transport of E1 and E2 out of the ER. In the presence of monensin, a swollen Golgi morphology was observed (Fig. 5n, p), along with a diffuse E2 and E1 distribution in the cytoplasm (Fig. 5m, o). Capsid protein was distributed in the cytoplasm in BFA- or monensin-treated cells (Fig. 5k, q). No cell-surface fluorescence was detectable in BFA- or monensin-treated cells (Fig. 5l, r).

**Effect of BFA and monensin on RV assembly and release**

To investigate the effects of BFA and monensin on RV assembly and release, we first monitored the release of virus particles from the infected cells into the culture medium. Vero cells were infected with RV at an m.o.i. of 10, labelled with [35S]methionine for 1 h at 24 h p.i. and incubated with medium containing BFA or monensin for 12, 20, 28 and 36 h. Radiolabelled virus particles in the medium were precipitated with polyethylene glycol and...
resuspended in RIPA buffer. Viral structural proteins were immunoprecipitated with human anti-RV serum and analysed by SDS–PAGE (Fig. 6). In control cells, the amount of viral structural proteins (C, E2 and E1) increased with time during the chase period (Fig. 6), indicating that virus particles were accumulating and that virus was steadily assembled and released from the cells. In BFA- or monensin-treated cells, no viral structural proteins were detectable until 36 h post-labelling (Fig. 6). Thus, BFA and monensin blocked RV release from the cells during early stages of the chase. After a 36 h chase period, the viral proteins detected may be due to cell lysis in BFA- or monensin-treated cells (Fig. 6).

To further address this question, a one-step growth experiment was performed with RV-infected Vero cells treated with BFA or monensin. BFA or monensin were added to the medium 8 h p.i. and maintained for 60 h. Cell-associated virus and virus in the medium were titred for infectivity. In control cells, intracellular and extracellular viruses reached a titre of about $5 \times 10^7$ p.f.u./ml at 48 h p.i., with a slightly higher extracellular than intracellular titre (data not shown). Titres of about $2 \times 10^5$ p.f.u./ml of cell-associated viruses were found in BFA- or monensin-treated cells, indicating that BFA and monensin dramatically reduced virus assembly in infected cells. Titres of extracellular virus from BFA- or monensin-treated cells represented only 0.1% of the total virus (data not shown). Thus, BFA and monensin effectively inhibited virus release from infected cells.

**Discussion**

Enveloped viruses require their host’s secretory pathway for virus assembly and release from the cells. The rate of intracellular transport and processing of viral proteins may play an important role in controlling the efficiency of virus maturation, particularly for viruses that are assembled at the cell surface. Mutations that impair the transport of viral proteins to appropriate cellular compartments have been found to reduce significantly the formation of infectious virus particles (Haggerty et al., 1991). In the Togaviridae family, RV differs from the alphaviruses by its long latency period, slow replication and low virus yield in cultured cells (reviewed by Porterfield et al., 1978), although both share similar virion structure as well as strategies for viral gene expression. The transport rate of RV glycoproteins in transfected cells is low compared to that of the envelope glycoproteins of alphavirus which have a $t_{1/2}$ of less than 30 min. In cells transfected with RV E2 cDNA, about 50% of E2 contains endo H-resistant sugar moieties after a 4 h chase period and resistance does not reach 80% after an 8 h chase (Qiu et al., 1992), indicating that E2 moves slowly from the ER to the Golgi stack. Immunofluorescence studies showed that the majority of RV glycoproteins E1 and E2 concentrate in the ER–Golgi region, suggesting that virus budding may occur at internal membranes.

Employing both O-glycosidase and endo F/PNGase to remove O- or N-linked oligosaccharides on E2, we found that the 37 kDa E2 contained only N-linked endo H-sensitive carbohydrates whereas the 42 kDa species bore both N- and O-linked glycans. The endo H-resistant sugar moieties present on the 42 kDa protein were due to the addition of O-linked glycans. These results suggest that E2 is first synthesized and processed as a 37 kDa protein in the ER with only N-linked high-mannose sugars. It is then transported to the Golgi where O-linked glycosylation occurs. Thus the acquisition of O-linked glycans can be used to monitor the transport of E2 to the Golgi complex. In BFA-treated cells, only the 42 kDa E2 protein containing both N- and O-linked sugars was observed (Figs 1 and 2). This probably resulted from the addition of O-linked glycans to the 37 kDa protein by the Golgi O-linked glycosylation enzymes which were brought back into the ER by BFA (Lippincott-Schwartz et al., 1989). In the presence of BFA, the addition of O-linked glycans to E2 took place quickly, whereas the maturation of N-linked sugars to complex form was
slow, as judged by the fact that complete endo H-resistant E2 was observed only after a 4 h chase (Fig. 1c). Although monensin appeared to abolish O-linked glycosylation in some viral glycoproteins (Collins & Mottet, 1992; Ogura et al., 1991), we found that E2 from monensin-treated cells possessed some O-linked glycans.

The intracellular distribution of RV structural proteins was dramatically altered in cells treated with BFA or monensin. In BFA-treated cells, the Golgi complex was disassembled (Fig. 5h, j). A dilated Golgi morphology was found in monensin-treated cells (Fig. 5n, p). The consistent co-localization of RV membrane glycoproteins with the Golgi markers suggests that RV envelope glycoproteins interact strongly with Golgi macromolecules. Recently, it has been shown that unassembled subunits of RV E1 glycoprotein are arrested in monensin-treated cells after examining all of the sections (data not shown). The results from the morphological analysis were consistent with a significant decrease in virus titre in BFA- or monensin-treated cells. The reduction of intracellular virus assembly in BFA- or monensin-treated cells could be due to blockade of glycoprotein transport to the site of envelopment or the disruption of vesicular structure that may be required for efficient virus assembly. Taken together, the results presented in this study suggest a correlation between intracellular localization of RV structural proteins and the site of RV assembly. Studies are in progress to examine the maturation of RV membrane proteins during transport from the Golgi apparatus to the cell surface and its possible role in RV assembly.

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