MOLECULAR BIOLOGICAL STUDIES OF RUBELLA VIRUS

STRUCTURAL PROTEINS

by

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We accept this thesis as conforming

to the required standard

The University of British Columbia

June, 1994

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The University of British Columbia
Vancouver, Canada

Date Oct. 10, 1994
Abstract

Rubella virus (RV) is a small enveloped RNA virus in the Togaviridae family. The virion contains three structural proteins, a capsid protein (C) associated with the genomic RNA to form the nucleocapsid and two membrane glycoproteins, E1 and E2. The RV structural proteins are translated as a polyprotein precursor p110 (NH₂-C-E2-E1-COOH) from a RV-specific 24S subgenomic RNA and derived by posttranslational processing of p110.

The role of N-linked glycosylation of E1 and E2 on their respective biological functions has been studied by expressing glycosylation mutants of E1 and E2 generated by oligonucleotide-directed mutagenesis on coding cDNA. Expression of the E2 mutant proteins in COS cells indicated that removal of any of the glycosylation sites resulted in slower glycan processing, lower protein stability and aberrant disulfide bonding of the mutant proteins, with the severity of defect depending on the number and location of deleted carbohydrate sites. Expressed E1 glycosylation mutant proteins from vaccinia recombinants were recognized by a panel of E1-specific monoclonal antibodies, indicating that carbohydrate side chains on E1 are not involved in the constitution of epitopes recognized by these monoclonal antibodies. All the E1 glycosylation mutants were capable of eliciting anti-RV E1 antibodies in immunized mice; however, only the single glycosylation mutants were found to be capable of inducing viral neutralizing antibodies, suggesting that carbohydrates on E1 are important for maintaining proper protein folding and epitope exposure.

Assembly of RV was found to be independent of the genomic RNA but strictly dependent upon the co-expression of C, E2 and E1, in stable cell lines expressing RV structural proteins. Assembly and release of RV virion was dramatically reduced in RV-infected cells treated with two Golgi transport inhibitors, brefeldin A and monensin, although there was no significant alteration for the expression and processing of the structural proteins. My finding indicates that stable association of RV E1 and E2 with the intact Golgi complex is essential for efficient RV assembly.
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<td>Description</td>
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</tr>
<tr>
<td>SV40</td>
<td>Similian virus 40</td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>tunicamycin</td>
<td></td>
</tr>
<tr>
<td>TRICT</td>
<td>tetramethylrhodamine isothiocyanate</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>trishydroxymethylaminomethane</td>
<td></td>
</tr>
<tr>
<td>VLP</td>
<td>virus-like particle</td>
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</tr>
<tr>
<td>VN</td>
<td>viral neutralizing</td>
<td></td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
<td></td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
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Lastly to my wife for her understanding throughout.

This thesis is dedicated to my parents.
1. INTRODUCTION

This introduction contains three subsections: an overview of biosynthesis and biological functions of asparagine-linked (N-linked) glycosylation on viral glycoproteins, recent progress in viral glycoprotein targeting and virus assembly, and a review of rubella virus biology. The rationale and objectives of this thesis will conclude the chapter.

1.1. N-linked glycosylation of viral glycoproteins

N-linked glycosylation is one of the most common post-translational modifications of proteins in the exocytic pathway of eukaryotic cells. Animal viruses utilize host-cell glycosylation machinery to synthesize and process oligosaccharides attached to viral glycoproteins. The expression of viral antigens in cells has proven to be a useful system for studying the stepwise events in glycan processing and intracellular transport along the exocytic pathway. From a large number of viral glycoproteins studied so far, none of the carbohydrate structures identified is unique to viral glycoproteins; they are also present in a variety of other membrane and secretory glycoproteins. However, the impact of N-linked glycosylation on conformation and subsequently on biological functions of viral glycoproteins varies with the protein in question.

1.1.1. Structure and biosynthesis of N-linked carbohydrate on viral glycoprotein

1.1.1.1. Transfer of oligosaccharide from a glycan-lipid precursor

All of the N-linked carbohydrates on viral glycoproteins is synthesized and processed by host cell enzymes following the general pathway for this class of glycans (for review see Kornfeld and Kornfeld, 1985). As the first step in the biosynthesis of glycoproteins, a core structure
(Glc$_3$Man$_9$GlcNAc$_2$) is assembled on the lipid carrier dolichol phosphate in the lumen of the endoplasmic reticulum (ER). Upon the translocation of newly synthesized polypeptide into the ER, the oligosaccharide is transferred, by oligosaccharide transferase, to the asparagine residues of polypeptides at the sequence Asn-X-Ser/Thr where X can be any amino acid except proline (Fig.1) (Kornfeld and Kornfeld, 1985). The oligosaccharide is covalently attached to the asparagine residues with a N-glycosidic linkage. A survey of protein sequences has revealed that not all the potential N-linked glycosylation sites are glycosylated (Kornfeld and Kornfeld, 1985). A properly oriented and accessible Asn-X-Ser/Thr motif in the protein is believed to play a major part in determining the efficiency of glycosylation.

1.1.1.2. Sequential processing of N-linked oligosaccharide

Once the oligosaccharide becomes polypeptide bound, it undergoes a series of trimming reactions by glycan modifying enzymes in the secretory pathway (Fig.1) (for review see Kornfeld and Kornfeld, 1985). Three glucose residues are first removed in the ER by glucosidases, followed by the removal of mannose residues by ER mannosidase or by α-mannosidase I present in the Golgi cisternae. At this stage, the oligosaccharide intermediate containing 5 to 8 mannoses is recognized by the Golgi enzyme, acetylglucosidase. This enzyme catalyzes the addition of an acetylglucoside residue to the free mannose linked α1-3 to the mannose residues. Afterward, two more mannose residues are removed by Golgi mannosidase II, leaving a free mannose for the further addition of acetylglucoside. After the acetylglucoside is added, the oligosaccharide becomes biantennary and is subjected to further addition of residues of galactose, fucose or sialic acid, catalyzed by glycosyl-transferases in the trans Golgi or trans-Golgi network, completing
Fig. 1. Processing of N-linked oligosaccharides to a representative biantennary complex structure. The scheme depicts the processing from the transfer of Glc₃Man₉GlcNAc₂ from its dolichol pyrophosphoryl derivative to the nascent polypeptide chain still bound to the ribosome, followed by processing reactions in the ER and GC. Oligosaccharide processing enzymes are listed above the line; the reaction they catalyze is depicted below the line (except for the alternate processing reaction, 3a). The subcellular localization of processing events are depicted by the brackets. Structures susceptible or resistant to digestion by endo H, an enzyme frequently used as a diagnostic test for processing to complex structures, are indicated. Symbols: □, glucose; ○, mannose; ■, N-acetylglucosamine; ●, galactose; ▽, fucose; ◆, sialic acid. (Moreman and Touster, 1988)
the assembly of the complex type oligosaccharide. Depending on the accessibility to glycan modifying enzymes and cell types, the extent of processing can be varied for different glycoproteins (for review see Klenk, 1990) or for oligosaccharides at different sites on the same protein species (Pollack and Atkinson, 1983), and this may explain the vast diversity of glycan differentiation in viral glycoproteins.

1.1.1.3. Characterization of the structure of N-linked glycans

The extent of N-linked oligosaccharide processing can be monitored using glycosidase digestion or lectin binding assays (Montreuil et al., 1986). Glycosidases are excellent tools to elucidate the primary structures of glycans by sequential degradation of oligosaccharides bound to the polypeptide backbone. Basically two types of enzymes are used: exoglycosidases hydrolyse glycosidic bonds of monosaccharides in terminal non-reducing positions and may achieve a stepwise degradation of the glycans; endoglycosidases hydrolyse internal glycosidic bonds. Each endoglycosidase usually recognizes a certain type of glycosidic linkage (Montreuil et al., 1986) (Table 1). By analysing sugar moieties that are liberated from the protein backbone with paper chromatography or FPLC, the structure of N-glycans on a protein can be defined.

Lectins are sugar-binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates (Lis and Sharon, 1984). Lectins are powerful tools for characterizing structure of oligosaccharides on glycoproteins because they bind with high specificity to certain types of glycoconjugates (Montreuil et al., 1984). Practically, immobilized lectins are widely used for isolating sugar components whereas fluorescence reagent-conjugated lectins are used for visualizing subcellular compartmentation.
1.1.2. Approaches for functional analysis of N-linked glycosylation

Several approaches have been used to define the functional roles of N-linked carbohydrate addition in cells. These include the use of agents that interfere with glycosylation (for review see Elbein, 1987), elimination of each N-linked glycan addition site on DNA or cDNA by oligonucleotide-directed mutagenesis and the use of glycan-processing-deficient cell lines.

Tunicamycin, a nucleoside analog, is the most widely used glycosylation inhibitor (Elbein, 1987). This antibiotic inhibits the transfer of GlcNAc-1-P from UDPGlcAc to dolichol phosphate (Fig.1). Since this step is the first in the lipid-linked pathway, tunicamycin treatment results in the synthesis of proteins that lack N-linked glycosylation (Elbein, 1987). Drugs that inhibit specific steps in the processing pathway have become available and have been extensively used in the study of the role of oligosaccharide processing intermediates in biological functions and transport of glycoproteins (Elbein, 1987). Furthermore, compounds such as brefeldin A (Fujiwara et al., 1988) and monensin (reviewed by Mollenhauer et al., 1990) which disrupt vesicular structures of cells and thus interfere with the normal distribution of resident glycan processing enzymes, have also been widely used in analyzing the effect of aberrantly processed glycans on the transport and function of glycoproteins.

While treatment with the above mentioned drugs may lead to altered cell metabolism (Elbein, 1987; Mollenhauer et al., 1990), the elimination of glycosylation sites on coding DNA or cDNA of a protein by site-directed mutagenesis has proven to be a valuable method to analyze the influence of carbohydrate site chain addition on glycoproteins in cells under normal growth conditions, especially when proteins with more than one N-linked glycosylation site are being examined. Expression and characterization of mutant viral glycoproteins with partial or
Table 1. Specificity of glycosidases used in this study (Montreuil et al, 1986)

<table>
<thead>
<tr>
<th>Enzyme Type</th>
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<tr>
<td>Endoglycosidase H</td>
<td>(Man)$_n$—Man</td>
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</tr>
<tr>
<td></td>
<td>z-Man—GlcNAc-1—GlcNAc-Asn</td>
</tr>
<tr>
<td></td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>x—Man</td>
</tr>
<tr>
<td></td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>y</td>
</tr>
<tr>
<td></td>
<td>Active on N-linked oligosaccharides of glycopeptides. Enzyme cleaves only high mannose structures [n=2-150, x=(Man)$_{1,2}$, y and z=H] or hybrid structures (n=2, x and/or y =NANA-Gal-GlcNAc or similar, z=H or GlcNAc).</td>
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<tr>
<td>Glycopeptidase F</td>
<td>x</td>
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<td></td>
<td>\</td>
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<tr>
<td></td>
<td>w—Man</td>
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<tr>
<td></td>
<td>\</td>
</tr>
<tr>
<td></td>
<td>u—Man—GlcNAc—GlcNAc-1—Asn</td>
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<td></td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>y—Man</td>
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<td></td>
<td>z</td>
</tr>
<tr>
<td></td>
<td>Active on N-linked oligosaccharides of glycopeptides. Enzyme cleaves high mannose structures (w, x and y= one or more Man residues, u and z=H) or hybrid structures (w and x=Man, y and/or z=NANA-Gal-GlcNAc or similar, u=H or GlcNAc) or complex structures (y and w=NANA-Gal-GlcNAc or similar, x and z=H, NANA-Gal-GlcNAc or similar, u=H or GlcNAc).</td>
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<tr>
<td>Neuraminidase</td>
<td>NANA$\alpha$2-1—X or NGNA$\alpha$2-1—X</td>
</tr>
<tr>
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<td>Enzyme cleaves terminal sialic acid residues which are $\alpha$2,3-, $\alpha$2,6- or $\alpha$2,8-linked to: Gal, GlcNAc, GalNAc, NANA, NGNA, oligosaccharide, glycolipid or glycoprotein.</td>
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complete depletion of N-linked glycosylation sites have provided greater understanding of the function of N-glycans.

A large number of cell lines have been isolated that are deficient in glycan processing enzymes. Infection of these cells with viruses and the analysis of protein expression, processing and virus production have yielded a great deal of useful information about the function of oligosaccharide side chains on proteins (Kennedy, 1974; Schlesinger et al., 1976; Hsieh and Robbins, 1984).

1.1.3. Biological functions of N-linked glycosylation

N-linked glycosylation influences a number of properties of proteins, including stability, intracellular transport, biological activity and antigenicity (for review see Olden et al., 1982; Klenk, 1990). There are no general rules existing regarding the consequences of changing the normal glycosylation pattern on a protein. Results from experiments using tunicamycin have indicated that the requirement for glycosylation is intrinsic to a given protein (Olden et al., 1982). Furthermore, in some cases, the biological effect of N-linked glycosylation depends on the particular site within the polypeptide chain.

1.1.3.1. Role of carbohydrate in initiating protein folding and maintaining protein stability

One of the most important functions of N-linked glycosylation is to initiate and maintain protein folding into its proper configuration. N-linked sugars are added co-translationally to the polypeptide chain as the consensus sequences emerge on the luminal side of the ER membrane, prior to or during folding. Inhibition of N-linked glycosylation, using tunicamycin or elimination
of N-linked glycosylation sites by mutagenesis, leads to misfolded proteins, such as aggregated or disulfide cross-linked complexes (Machamer et al., 1985; Rose and Doms, 1988; Ng et al., 1990; Vidal et al., 1992). It is believed that the hydrophilic oligosaccharides render folding intermediates more soluble and less likely to form irreversible aggregates (Rose and Doms, 1988).

The importance of individual N-linked oligosaccharide side chains depends on the protein in question as well as the location of the glycosylation site within the protein. Elimination of some sites may result in little or no defect whereas others may be essential for correct folding and stability (Ng et al., 1990; Sodora et al., 1991; Roberts et al., 1991; Pique et al., 1992). Elimination of individual glycosylation sites can also lead to the generation of temperature-sensitive (ts) mutants (Gallagher et al., 1988; Machamer and Rose, 1988; Ng et al., 1990) and the effect of such elimination can be additive and strain-specific (Machamer et al., 1985; Gallagher et al., 1992).

Oligosaccharides on glycoproteins are believed to provide protection against protease attack (reviewed by Olden et al., 1982). After treatment with glycosidases to remove glycans on glycoproteins, the polypeptide backbones become more susceptible to protease digestion (Olden et al., 1982). On the other hand, a higher turnover rate has been observed for many glycosylation mutant proteins and for glycoproteins from cells treated with glycosylation inhibitors (Ng et al., 1990; Sodora et al., 1991; Roberts et al., 1991). The deleterious effect of elimination of glycosylation on protein stability parallels that of protein folding (Ng et al., 1990; Sodora et al., 1991). Proteins misfolded due to inhibition of glycosylation by tunicamycin or deletion of glycosylation sites have a low solubility and tend to form aggregates in the ER and be degraded.
1.1.3.2. Role of carbohydrate in facilitating intracellular transport and processing

It is now clear that carbohydrate itself does not determine the subcellular destination of viral glycoproteins. However, glycosylation greatly enhances the movement of many glycoproteins out of the ER, although this often results through effects on protein folding. It is possible though, that oligosaccharides could have the effect of increasing transport rates by generating affinities towards enzymes sequestered in the exocytic pathway.

The requirement for carbohydrate in the transport of membrane and secretory proteins is not universal and is highly protein-specific (reviewed by Olden et al., 1982). Some proteins are transported and function normally when glycosylation is inhibited with tunicamycin, whereas others exhibit folding defects, frequently resulting in protein aggregation in the ER or rapid degradation (Machamer et al., 1985; Rose and Doms, 1988; Hurtley and Helenius, 1989; Ng et al., 1990). It has also been shown that the arrest of transport of misfolded viral protein in the ER can be rescued at a lower temperature (Gallagher et al., 1988; Machamer and Rose, 1988; Ng et al., 1990), which supports the concept that retention in the ER is due to protein misfolding. Addition of supernumerary carbohydrate side chains, (e.g. for VSV G and influenza virus HA), can also have the similar effect, disrupting folding and transport (Doms et al., 1988; Gallagher, et al., 1988; Machamer and Rose, 1988a). However, some degree of flexibility has been observed in the position on the polypeptide backbone at which carbohydrate chains are required (Doms et al., 1988, Machamer and Rose, 1988b). A single amino acid substitution of VSV G protein has been found to eliminate the stringent carbohydrate requirement for cell surface expression of the
mutant VSV G protein (Pitta et al., 1989).

Carbohydrates may affect in two ways, the proteolytic processing of viral precursor glycoprotein: as a general requirement for proper protein folding and subsequently transport to the site of cleavage, or as a factor modulating protein configuration within or adjacent to the cleavage site for the access of protease. Inhibition of N-linked glycosylation inhibits the processing of Sindbis virus envelope protein E2 (Leavitt et al., 1977) and the Newcastle Disease virus F glycoprotein (Morrison et al., 1985) due to failure in transport of nonglycosylated proteins to the Golgi complex for cleavage. Using oligonucleotide-directed mutagenesis, others have found the site-specific influence of glycosylation on cleavage of the CK/Penn strain of avian influenza virus HA (Deshpande et al., 1987), Friend murine leukemia virus envelope protein (Kayman et al., 1991) and measles virus fusion protein (Alkhatib et al., 1994).

1.1.3.3. Role of carbohydrate in modulating biological activities of proteins

The envelope glycoproteins of animal viruses may be involved in the attachment of virus to the host cell receptor, and fusion between the virus envelope and the cell membrane; they also serve as the target for host immune surveillance (discussed below). The importance of N-glycosylation of viral glycoproteins in receptor binding has been illustrated in some detail for human immunodeficiency virus type I (HIV-1) envelope glycoprotein gp120 (Fenouillette et al., 1990). Deglycosylation of HIV-1 gp120 by glucosidase digestion results in a less than 10-fold reduction of the ability to bind to CD4, the cellular receptor for HIV-1 (Fenouillet et al., 1990). Deglycosylation also significantly reduces but does not abolish, HIV-1 binding to and infectivity of CD4+ cells (Fenouillette et al., 1990). Anticarbohydrate monoclonal antibodies (mAbs) can
block infection by cell-free virus as well as inhibiting syncytium formation, probably through steric hindrance (Hansen et al., 1990). However, nonglycosylated HIV-1 gp120 synthesized in the presence of tunicamycin fails to bind to CD4 (Li et al., 1993). These data suggest that glycosylation of gp120 is essential to create a conformational epitope to which CD4 binds, but is not directly involved in CD4-binding.

In contrast, treatment of respiratory syncytial virus with N-glycanase and O-glycosidase under mild conditions to remove readily accessible carbohydrate from respiratory syncytial virus glycoprotein results in a significant loss of virus infectivity (Lambert, 1988), suggesting that carbohydrate exerts a considerable influence on the attachment and/or penetration function of the viral glycoproteins. Similarly, enzymatic removal of N-linked oligosaccharide from hemagglutinin-neuraminidase (HN) glycoprotein of human parainfluenza virus type 1 leads to a change in the interaction of HN with the host receptor, sialic acid (Gorman et al., 1991).

Site-specific contribution of N-linked glycosylation on a viral protein towards their biological activities has been studied in several viruses employing mutagenesis approach. Mutations at particular sites in envelope glycoproteins have been found to be responsible for loss of infectivity of Friend murine leukemia virus (Kayman et al., 1991), and a decreased induction of polykaryon formation in measles virus (Alkhatib et al., 1994) and HIV-1 (Dedera et al., 1992). Finally, lack of glycosylation at a conserved site in influenza virus neuraminidase has been found to confer strain-specific neurovirulence in mice (Li et al., 1993).

1.1.3.4. Role of carbohydrate in influencing antigenic properties

Carbohydrates on viral glycoproteins can modulate the antigenicity and immunogenicity of viral
glycoproteins, directly or indirectly. The indirect influence refers to the fact that oligosaccharide can promote and maintain the correct folding of viral glycoproteins and thus stabilize the epitopes or facilitate epitope exposure. It is often found that synthetic peptides bearing neutralizing epitopes of viral glycoproteins or viral glycoproteins expressed from E.coli fail to induce neutralizing antibodies, suggesting that lack of glycosylation may reduce the immunogenicity of proteins. Since the contribution of each glycosylation site within a protein possessing multiple glycosylation sites may be different (see 1.1.3.1), the site-specific effect of glycosylation on the immunoreactivity has been characterized for a number of viral glycoproteins. Using site-directed mutagenesis to alter N-linked glycosylation sites, it has been found that one glycosylation site on bovine herpesvirus type 1 glycoprotein gIV is important for its immunoreactivity (Tikoo et al., 1993). Mutant proteins lacking glycans at residue 102 show altered reactivity with conformation-dependent gIV-specific mAbs and also induce significantly lower neutralizing antibody responses than wild-type (Tikoo et al., 1993).

On the other hand, evidence has been obtained that carbohydrates can shield antigenic sites from immune recognition by steric hindrance. Addition of carbohydrate side chains at novel sites on influenza virus hemagglutinin results in the shielding or disruption of functional epitopes on the surface of hemagglutinin (Gallagher, et al., 1988). One of the Sindbis virus neutralization escape mutants selected with mAbs shows a codon change which results in the gain of a new glycosylation site at amino acid residue 203 of the E2 protein (Davis et al., 1987). A more common approach utilized in studying carbohydrate shielding of epitopes on viral glycoproteins is the use of glycosidases to remove glycans from expressed glycoproteins or from assembled virions, and analysis of the antibody binding activity as well as the immunogenicity of these
deglycosylated proteins or virions. Deglycosylation does not affect the binding of Rauscher leukemia virus envelope glycoproteins to neutralizing antibodies from sera; and in fact, deglycosylated virions induce a faster neutralizing antibody response than that of untreated control virus (Elder et al., 1986). The immunoreactive conformation of envelope glycoproteins of HIV-1 remains unaltered after deglycosylation (Ferouillet et al., 1990). However, rabbits immunized with these deglycosylated glycoproteins produce lower viral neutralizing (VN) antibodies that inhibit HIV-1 infectivity or syncytium formation in infected cells (Benjouad et al., 1992).

Besides total removal of glycans from glycoproteins, partial removal of sugar moieties from carbohydrate side chains or inhibition of oligosaccharide processing have also been found to interfere with the fine conformation of domains in the polypeptides within or adjacent to glycosylation sites and to result in an altered antigenic properties. The glycoprotein gIV of bovine herpesvirus 1 expressed from recombinant baculovirus infected insect cells, which are devoid of sialyl transferases for the addition of terminal sialic acid to the N-glycans, reacts less efficiently with mAbs that recognize conformation-dependent epitopes, and induces lower overall or neutralizing antibody titres, than the protein from virion grown in mammalian cells (van Drunen Littel-van den Hurk, et al., 1991). In cells treated with N-methyl-1-deoxynojirimycin, an inhibitor of \( \alpha \)-glucosidase, the normal carbohydrate trimming is inhibited. Under these conditions, SFV E2 protein expressed from SFV-infected chicken cells contains three additional glucose residues in the oligosaccharide side chain and has a significantly changed antigenicity (Kaluza et al., 1980).

Carbohydrates can serve directly as targets for recognition by the immune system.
Because of their host specificity, carbohydrate epitopes are responsible, by a mechanism of molecular mimicry, for cross-reactivities with host components or with other viral glycoproteins carrying similar oligosaccharides. This has been well documented in the case of influenza virus (Klenk, 1990). Carbohydrate epitopes are rare; more frequently, the sugar moieties are part of the constitution of conformation-dependent epitopes on viral glycoproteins. Depletion of N-linked glycan either by changing the consensus sequence for N-glycosylation or by total inhibition of N-glycosylation using tunicamycin results in the reduced reactivity of bovine herpesvirus 1 gIV to mAbs that recognize conformation-dependent epitopes but not those which react to linear epitopes (Tikoo, et al., 1993). Immunization of animals with mutant protein in which oligosaccharide side chains involving conformation-dependent epitopes are deleted, results in no significant difference in total antibody responses; however, the neutralizing titre of the antibodies is much lower (Tikoo, et al., 1993).
1.2. Targeting of viral membrane glycoproteins and virus assembly

1.2.1. General concepts

1.2.1.1. Assembly of enveloped viruses

Enveloped viruses package their genomes within a protein shell, and this nucleocapsid (or core structure) is then enveloped by a lipid bilayer at the final step of virus maturation, the budding process, during which the nucleocapsid core extrudes itself through a certain region of the cellular membrane. The envelope of viruses is made up of a regular lipid bilayer derived from, and similar in structure and composition to, one of the host cell membranes. However in this process, the host membrane proteins are effectively excluded and replaced with virus-specific membrane proteins (Suomalainen et al., 1992). The mechanism of virus budding is still largely unknown. It is believed that the interaction between the nucleocapsid and viral membrane glycoproteins, in most cases, is the driving force for virus assembly. Accumulation of viral proteins within a specific subcellular compartment may provide ground for such an interaction and may, at least partially, determine the site of virus maturation.

In general, the unambiguous proof of virus maturation at any particular subcellular site is provided by the demonstration of electron microscopic profiles of virus particle accumulation and budding. Virus assembly at almost all cellular membrane structures, plasma membrane (PM), Golgi complex (GC), endoplasmic reticulum (ER) and inner nuclear membrane has been reported. By comparing the electron microscopic profiles of virus budding with data from light- and electron-microscopic immunolocalization of the distribution of viral glycoproteins as well as the nucleocapsid, a strong correlation between the site of virus budding and glycoprotein targeting
has been found for a number of viruses.

1.2.1.2. Targeting of viral envelope glycoproteins

Viral proteins destined for the plasma membrane follow the general secretory pathway also utilized by host cell plasma membrane proteins. In fact, viral spike proteins have been instrumental in dissecting the various steps involved in this exocytic pathway. The organelles within this system include the rough and smooth ER, the cis-medial- and trans-Golgi, the trans-Golgi network (TGN), secretory vesicles and granules, and the PM (reviewed by Dunphy and Rothman, 1985). The ER represents the point of entry for the proteins that will traverse this complex organellar pathway (reviewed by Pfeffer and Rothman, 1987). Proteins synthesized on the polysomes associated with the ER membrane, are cotranslationally inserted into the ER due to the presence of a signal sequence (Singer et al., 1987). During and after the process of translocation itself, components of the ER play a variety of roles in catalyzing posttranslational modifications such as proteolysis and N-linked glycosylation of the extruded proteins. In addition it has become increasingly clear that molecules residing within the lumen of the ER assist in the correct folding of translocated polypeptides and their assembly into oligomeric complexes (Ng et al., 1990; Earl et al., 1991). The attainment of a correct structure appears to be critical for transport out of the ER to the Golgi, and may in fact be the rate-limiting step in the process (see 1.1.3.1 of the introduction) (reviewed by Rose and Doms, 1988). Proteins normally targeted to the PM exit the ER and move to the Golgi cisternae via vesicle-mediated membrane fusion. The GC is a set of subcompartments that comprises at least three biochemically distinct units (reviewed by Dunphy and Rothman, 1985), based on the enzymes that transform N-linked
oligosaccharide chains into their mature form. As glycoproteins are transported from cis through medial to trans Golgi, carbohydrate modifications are carried out by resident enzymes. A large variety of other processes such as O-linked glycosylation, acylation, sulphation, and proteolytic cleavage/activation also occur in the GC. The majority of membrane-spanning proteins are transported to the PM in a constitutive, unregulated fashion, whereas secretion of soluble proteins involves the TGN, which is a branchpoint directing the final destination of proteins (Pfeffer and Rothman, 1987).

Not all the proteins that enter the secretory pathway end up at the cell surface; a number of cellular and viral glycoproteins are retained in one of the subcellular compartments of the exocytic pathway (the ER, the GC or the TGN). The intracellular retention of glycoproteins is believed to be mediated by "retention signals" located in the primary structure of proteins (Lewis and Pelham, 1989; Swift and Machamer, 1991). Many ER-resident proteins bearing an amino acid motif of Lys-Asp-Glu-Leu (KDEL) or similar sequences interact with the KDEL receptor which normally resides in the cis Golgi cisternae (Lewis and Pelham, 1989). Ligand binding induces a change of conformation in the KDEL receptor and results in the retrograde movement of the receptor-ligand complex back to the ER (Lewis and Pelham, 1991). The Golgi retention signal has been found to be located in the transmembrane domains of proteins (Swift and Machamer, 1991) while the cytoplasmic tail has been shown to be important in retention in the TGN.

In most studies of protein targeting, the criteria of cell surface expression of glycoproteins is defined using immunofluorescence techniques while subcellular localization of proteins can be determined using light- and electron-microscopic immunolocalization.
Biochemical analysis such as analysis of the extent of N- or O-linked glycosylation, organelle-specific proteolytic processing and the oligomeric state of the proteins in question have also been extensively used to study protein transport.

1.2.1.3. Experimental approaches

Conventional electron-microscopic profiles of virus particle accumulation and budding of infected cells at different stages during virus replication provide adequate information on the site of virus budding. Recent developments in immuno-electron microscopic techniques allow the fine localization of virus-specified proteins in virus-infected cells. This methodology has been applied to transfected cells expressing viral protein from DNA or cDNA constructs derived from partial or intact viral genomes. Comparison of protein localization with or without virus assembly has shed light on which viral proteins determine the site of budding.

While morphological studies create a steady-state image of virus budding and virus particle accumulation, data from biochemical analysis have been proven to be very informative on the stepwise processes from the synthesis of viral proteins to their incorporation into virions. In such studies, two protein transport inhibitors, brefeldin A (BFA) and monensin have been extensively utilized. Monensin, a monovalent ion-selective ionophore, facilitates the transmembrane exchange of principally sodium ions for protons, which results in the neutralization of acidic intracellular compartments such as the trans Golgi apparatus and associated elements, combined with a disruption of the normal functions of these compartments. Late Golgi processing events such as terminal glycosylation and proteolytic cleavages are most susceptible to inhibition by monensin (reviewed by Mollenhauer et al., 1990).
BFA, a fungal metabolite, is another compound which has been extensively utilized in studying ER-Golgi trafficking in recent years. At low concentrations (1μg/ml) this lipophilic molecule retards protein secretion, apparently acting specifically on transport from the ER to the Golgi (Misumi et al., 1986). At an intermediate concentration (2.5 μg/ml) the Golgi is disassembled, while at 10 μg/ml, transport is completely blocked and morphological changes including dilation of the ER as well as loss of the Golgi structure (Fujiwara et al., 1988, Misumi et al., 1986). At high concentration, BFA causes the movement of resident Golgi proteins back into the ER (Doms et al., 1989). This inhibitory effect of BFA is reversible. It should be noted that although these observations are applicable to most type of cells, the effect of BFA treatment in each cell type is cell-type specific.

1.2.2. Virus assembly at the PM

Most enveloped animal viruses (e.g. alphaviruses, arenaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, and retroviruses) acquire their envelope at the PM. After budding has been completed, virus particles are released directly into the extracellular space.

1.2.2.1. Morphogenesis and assembly

For virus maturation at the PM, in most cases the pre-assembled nucleocapsid is transported to the cell surface and interacts with viral glycoprotein(s) embedded in the PM, leading to formation and release of the virus particles. Matrix or membrane (M) proteins are involved, for some viruses, in this nucleocapsid-glycoprotein interaction. In the unusual case of lentiviruses, assembly of the core and virion occurs simultaneously, and does not require the envelope glycoprotein.
1.2.2.1.1. Assembly mediated by spike-nucleocapsid interactions

Alphaviruses are enveloped RNA viruses belonging to the Togaviridae family. The virions appear as essentially spherical, 60-65 nm diameter particles. The genome, a 49S RNA molecule with positive polarity, is encapsidated by a single species of capsid protein arranged in an icosahedral configuration. This nucleocapsid is enveloped by a lipid bilayer derived from the host cell plasma membrane. Projecting from the bilayer and embedded in it are the viral encoded glycoproteins designated E1 and E2. For alphaviruses replicating in vertebrate cells, the assembly of nucleocapsid takes place in the cytoplasm while the budding occurs primarily at the plasma membrane (Smith and Brown, 1977). Several ts mutants of alphaviruses have been isolated which have maturation defects associated with the spike proteins at the restrictive temperature and thus are defective in the release of infectious virions. In some ts mutants, the mutated spike proteins fail to be transported to the plasma membrane at the restrictive temperature, and electron microscopic (EM) analysis of these cells shows no budding structure under these conditions (Brown and Smith, 1975; Saraste et al., 1980; Smith and Brown, 1977). In one Sindbis virus ts mutant (ts20)-infected cells, the altered spikes are able to reach the cell surface, and in these cells, the plasma membrane is seen to be lined with nucleocapsids engaged in the budding process (Smith and Brown, 1977; Saraste et al., 1980). These results indicate that the nucleocapsid cannot bud from the cell without correct nucleocapsid-spike protein interaction at the cell surface. Amino acid substitutions in the cytoplasmic tail of E2 have been shown to lead to defects in virion assembly (Gaedigk-Nitschko and Schlesinger, 1991). Recently, using recombinant SFV genomes lacking the nucleocapsid protein gene or, alternatively, the spike genes, Suomalainen et al. (1992) demonstrated that virus release is strictly dependent on the
coexpression of the nucleocapsid and spike proteins and concluded that the budding of alphaviruses is mediated by nucleocapsid-spike interaction.

1.2.2.1.2. Matrix or membrane (M) protein mediated nucleocapsid-glycoprotein interaction
Rhabdoviruses, orthomyxoviruses and paramyxoviruses are negative-stranded RNA viruses. Although there are differences in their genomic organization (segmented or nonsegmented), virion structure (spherical or bullet-shaped) and gene expression strategy, they are similar in that virion maturation occurs at the cell surface and is mediated by respective M proteins. The ribonucleoprotein cores are assembled in the nucleus (orthomyxoviruses) or cytoplasm (rhabdoviruses and paramyxoviruses) and transported to a region of the plasma membrane which contains newly inserted but randomly distributed viral glycoproteins. The M proteins bridge the gap between the ribonucleoprotein core and the cytoplasmic extension of the glycoproteins, leading to the envelopment of nucleocapsid with glycoprotein-covered plasma membrane. For vesicular stomatitis virus (VSV), a rhabdovirus, the M (matrix) protein binds to progeny nucleocapsid and the nucleocapsid-M-protein complex migrates to the cell surface to initiate budding (Newcomb et al., 1982). In paramyxoviruses, the M (membrane) proteins aggregate in the inner aspect of the cell surface and noncovalently associate with glycoprotein. The complex ‘captures’ the newly arrived nucleocapsid to activate the budding process (reviewed by Dubois-Dalq et al., 1984).

1.2.2.2. Spike glycoprotein transport
Much of what is known about stepwise transport of glycoproteins from the site of biosynthesis to the cell surface has been learned from the study of some viral glycoproteins: VSV G protein,
influenza HA and alphavirus p62 and E1. Although the movement of these proteins from the ER to the cell surface is fast, with a $t_{1/2}$ of less than 30 minutes, they are subjected to a number of structural modulations. The lumen of the ER provides an environment optimized for protein folding and multi-subunit assembly. The ectodomain of the polypeptide must fold correctly in order to be transport competent (Earl et al., 1989). Protein misfolding induced by inhibition of glycosylation (by TM or mutagenesis) results in the aggregation and cross-linking of proteins by disulfide bonds (Hurtley et al., 1989; Machamer and Rose, 1988b). These defective proteins are retained in the ER and eventually degraded (Lippincott-Schwartz et al., 1988). Retention prevents delivery of nonfunctional viral membrane proteins to the site of virus budding. Movement from the ER to GC is the rate-limiting step in the exocytic pathway for viral membrane proteins that are targeted to the cell surface, whereas intra-Golgi transport is fast (Pfeffer and Rothman, 1987). Transport between Golgi compartments is a vesicular process during which proteins are exposed to the sequential action of N-linked glycosylation modification enzymes residing in each subdivision of the GC (Dunphy and Rothman, 1985). Compartmentalization is also important in that it allows protein cleavage/activation to occur at a specific stage of maturation within the Golgi, as in the case for the conversion of alphavirus p62 to E2 and E3 (Hakimi and Atkinson, 1982).

2.2.3. Studies with inhibitors, BFA and monensin

BFA and monensin effectively block the incorporation of the VSV G protein and E1 and E2 proteins of SIV into their respective virions as a result of the inhibition of transport of these
proteins to the plasma membrane. In the presence of BFA, the acquisition of endo H resistance by VSV G protein and the proteolytic conversion of SIV pE2 to E2 (a Golgi-specific event) are inhibited, suggesting that the transport of these envelope proteins is arrested in the ER (Oda, et al., 1990). In monensin-treated cells, fatty acid attachment to VSV G and SIV pE2, and the posttranslational removal of mannose residues from oligosaccharides on VSV G occurs normally, whereas proteolytic cleavage of SIV pE2 to E2 is inhibited, suggesting that monensin acts during late Golgi processing (Johnson and Schlesinger, 1980).

1.2.3. Viruses assembled in the GC

Members of the Bunyavirus family are the only viruses in which budding occurs for certain in the GC. Although there are a large number of viruses in this family, they share a similar general structure and site of maturation.

1.2.3.1. Morphogenesis and assembly

Bunyavirus particles are 90-100 nm in diameter and contain two membrane glycoproteins, G1 and G2. The internal protein N associates with RNA to form the nucleocapsid. EM studies show that virus particles mature intracellularly by budding into smooth vesicles in a perinuclear region and the budding structure is not observed at the PM. During Uukuniemi virus infection, both G1 and G2, as well as N, probably in the form of nucleocapsids, accumulate in the GC. The helical nucleocapsids appear to line up beneath the membrane of distended Golgi vesicles. As G1 and G2 accumulate in the GC, progressively more nucleocapsid seems to enter the GC region. Little if any N protein is seen associated with the ER or the PM. Thus specific interactions between
nucleocapsids and membranes containing viral glycoproteins exist only in the GC (Kuismanen et al., 1982, 1984).

1.2.3.2. Targeting of G1 and G2 to the GC

Based on primary structure deduced from cDNA sequences, G1 and G2 from most of the viruses in Bunyavirus family are type I membrane glycoproteins (Pettersson, et al., 1988). The oligosaccharides on G1 and G2 are found to be heterogeneously processed, as judged by endo H digestion and analysis of terminal glycans. The presence of immature glycans may reflect the site of maturation in the GC. In virus-infected cells or cells expressing viral protein from vaccinia recombinant virus, most of the glycoproteins accumulate in the GC and cannot be chased out from there. This strong retention of glycoproteins in the GC suggests that a retention signal may reside in either G1 or G2, or both.

1.2.3.3. Inhibitor studies, BFA and monensin

BFA treatment does not affect the assembly of intracellular infectious virus particles of Punto Toro virus but causes a rapid and dramatic change in intracellular distribution of G1 and G2 glycoproteins, from a Golgi pattern to an ER pattern (Chen et al., 1991). In contrast, budding of bunyanvirus is inhibited by the ionophore monensin (Cash 1982; Kuismane et al., 1985, Chen et al., 1991) whereas the association of the nucleocapsid with Golgi vesicles seems to be unaffected (Kuismane et al., 1985). This points to the possibility that the pH or the ionic milieu prevailing in the GC is critical for bunyavirus budding. Budding in the GC may thus be dependent not only on a certain concentration of glycoproteins, but also on a conformational
change of the glycoproteins induced by this milieu.

1.2.4. Virus assembly in the ER, the GC or a pre-Golgi compartment

Members of the coronaviridae family are enveloped RNA viruses that acquire their lipoprotein coats by budding at intracellular membranes (the ER, the GC, or in a compartment between these two organelles). Coronaviruses have a single species of coat protein, N protein, which is associated with the genomic RNA in the cytoplasm to form helical, loosely coiled nucleocapsids and two membrane glycoproteins M and S.

1.2.4.1. Morphogenesis and assembly

Coronavirus budding occurs at intracellular membranes between the rough ER and the Golgi apparatus. At the budding site, nucleocapsids align on the cytoplasmic side of smooth membrane in the ER, or the GC where viral glycoproteins have accumulated (Massalski et al., 1982). Budding at the cell surface has not been observed. In a detailed electronmicroscopic study of different types of cells infected with mouse hepatitis virus A59, slight differences in the sites of virus maturation were observed, either between the GC and the smooth perinuclear vesicles, or tubules in a pre-Golgi region (Tooze et al., 1984; Tooze and Tooze, 1985; Tooze et al., 1987). It appears that the location of coronavirus budding is at least in part determined by the host cell.

1.2.4.2. M protein targeting determines the site of coronavirus maturation

Amino acid sequences predicted from cDNA sequences reveal that coronavirus M glycoprotein is a type III membrane glycoprotein with multiple transmembrane domains (Mayer et al., 1988;
Based on the topological model and protease protection assays, it is proposed that most of the M protein is embedded in the membrane (Mayer et al., 1988). M protein contains either N- or O-linked oligosaccharides depending on the strain. Immunolocalization of M protein in coronavirus-infected cells or cells transfected with a cloned cDNA has indicated that it is predominantly found in smooth membranes of the pre-Golgi region, the GC and, later in infection, also in the ER (Rottiri and Rose, 1987; Machamer and Rose, 1987; Mayer, et al., 1988). Recent studies employing immuno-electronmicroscopic and recombinant DNA techniques have shown that the M protein is targeted to the GC through a "Golgi retention signal" localized in the first transmembrane domain of M protein (Machamer et al., 1990; Swift and Machamer, 1991; Machamer et al., 1993). This "retention signal" is sufficiently efficient to cause proteins normally targeted at the cell surface (e.g. VSV G) to be retained in the GC (Swift and Machamer, 1991).

The site of virus budding is determined by the subcellular localization of M protein, based on several lines of evidence. M protein is never transported beyond the trans Golgi to the PM, and no virus budding has been observed at the PM, although a large amount of S protein may accumulate at the PM (Mayer et al., 1988). In coronavirus-infected cells treated with tunicamycin, M protein and the nucleocapsid protein are normally incorporated into virions in the ER and Golgi without S protein (Holmes et al., 1981). In this respect, coronavirus M protein resembles the matrix or membrane proteins of paramyxovirus and rhabdovirus in function during virus assembly, although it is a glycoprotein.
1.2.4.3. Studies with monensin

Studies by Niemann et al., (1982) show that monensin does not interfere with coronavirus budding in the rough ER or Golgi, but it does inhibit virus release and fusion of infected cells. During monensin treatment, oligosaccharides on the S protein are shown to be resistant to endo H digestion but lack fucose, indicating that transport of the S protein is inhibited between the trans Golgi and the cell surface. The M protein incorporated into virions is devoid of carbohydrate, implying that the transport of M protein is also inhibited by monensin.

1.2.4. Closing remarks

In the case of viruses that bud at the plasma membrane, the viral glycoproteins are rapidly transported to the cell surface via the normal exocytic pathway (Stephens and Compans, 1988). By contrast, for most of the intracellular maturing enveloped viruses, at least one of the virus-specified glycoproteins is targeted to and accumulates in the budding compartment. Examples of such glycoproteins have been discussed earlier in this introductory section, e.g. coronavirus M protein (in a post-ER, pre-Golgi intermediate compartment, or GC) and bunyavirus G1 and G2 (GC). Thus, one important factor in determining the site of budding is clearly the targeting to and accumulation of viral glycoprotein in the compartment.

Viruses incorporate functional gene products into virions. The first step in this quality control mechanism is that newly synthesized viral glycoproteins must fold into a proper conformation to obtain transport competence. For proteins destined for the cell surface, this is the rate-limiting step in the exocytic pathway, during which they undergo a series of posttranslational modifications and become biologically functional upon reaching plasma
membrane. For proteins targeted to an intracellular compartment, however, correct protein folding enables proteins to exit the ER and to be retained in one of the compartments. Such retention may be due to 1) the presence of specific amino acid motif (linear or conformational) that constitute a "retention signal" (as in the case of coronavirus M glycoprotein); 2) lateral interaction between viral membrane glycoproteins that result in the formation of large aggregates that exclude them from transport vesicles; 3) association with macromolecules residing in a subcellular compartment via interaction with regions of the protein other than the "retention signal".

To date, little is known about the mechanism underlying virus budding, particularly for virus budding at the intracellular membrane. It is understandable that an important prerequisite for virus budding may be the need for a critical concentration of viral glycoproteins within the budding compartment. A good explanation is that normally a particular type of virus buds only in one of the subcellular compartments. Conformational changes in glycoproteins along the transport route induced by milieu (pH, ionic conditions) or posttranslational modifications (e.g. glycan processing) may facilitate the interaction of viral glycoproteins with nucleocapsids. Lastly, compartment-specific molecules may assist the budding process.
1.3. Rubella virus biology

1.3.1. Classification

Rubella virus (RV) is the sole member of the genus Rubivirus in the family Togaviridae (Porterfield et al., 1978). Based on morphological criteria, Togaviruses are defined as spherical, enveloped viruses with an icosahedral nucleocapsid. The genome is composed of a single infectious RNA molecule. Surrounding the nucleocapsid is the host cell-derived lipid bilayer containing viral membrane glycoproteins. Progress in the molecular characterization of viruses in the Togaviridae family has led to reclassification of these viruses on the basis of viral genome structure, organization, and gene expression. Under the current classification, the Togaviridae family consists of two genera: alphaviruses (arthropod-borne) and rubiviruses (non-arthropod-borne) (Francki et al., 1991). The two well-studied viruses, Sindbis virus (SIN) and Semliki Forest virus (SFV) are included in the alphavirus genus whereas RV is the only known member of rubivirus genus (Francki et al., 1991).

1.3.2. Clinical aspects

RV is the etiological agent of a relatively mild childhood disease known as German measles. RV infection in humans may be asymptomatic or can induce adenopathy, malaise, low grade fever, and exanthem. The most common complication following natural RV infection is transient joint involvement such as polyarthritis and arthritis.

The primary medical significance of RV infection is that the virus can cross the placenta and replicate in the fetus. Infants born to women infected during the first trimester of gestation
have a high incidence of birth defects, collectively known as congenital rubella syndrome (CRS). These include heart defects, cataracts, deafness, and mental retardation. Vaccination with live, attenuated virus has been successful in reducing the incidence of CRS. However, rubella-associated arthritis and the consequence of viral persistence in vaccinees resulting from RV vaccination remain major medical concerns (Chantler et al., 1982). Furthermore, RV infection has been linked to some chronic diseases, including autoimmune diseases (Wolinsky, 1990). Although the highest correlation between RV infection and chronic disease is found in the CRS population (Wolinsky, 1990), the association of RV persistence with arthritis (reviewed by Phillips, 1989) and multiple sclerosis (Nath and Wolinsky, 1990) has been suggested.

1.3.3. Morphology and morphogenesis

Early studies employing conventional electron microscopy of RV grown in BHK-21 cells indicated that RV virions are spherical, 60-70 nm in diameter, with a 30 nm electron dense core surrounded by an envelope (von Bonsdorff and Vaheri, 1969). These structures have been defined as the icosahedral nucleocapsid (the dense core) (Murphy et al., 1968) and lipid-bilayer (envelope associated with the hemagglutination activity) (Holmes et al., 1969). The mechanism of RV assembly and budding is largely unknown. Among reports on the RV budding site, there is an apparent discrepancy. In BHK-21 and Vero cells, intracellular maturation (in Golgi or vacuoles) and budding at the plasma membrane have been observed (Bardeletti et al., 1979; Payment et al., 1975). However, studies in which cells were infected with RV at high multiplicity of infection (MOI) and analyzed at different time intervals suggest that there exits a course of progression of RV maturation from the Golgi towards the plasma membrane (von Bonsdorff and Vaheri,
1969; Bardeletti et al., 1979).

**1.3.4. Nucleic acids and genome organization**

The RV genome, a single-stranded RNA molecule with a sedimentation coefficient of 40 S, is infectious (Hovi and Vaheri, 1970). RV-infected cells contain, in addition to the 40 S RV genome, an RV-specific RNA molecule which sediments at 24 S (Oker-blom et al., 1984). This subgenomic RNA is polyadenylated, capped, and identical to the 3' one-third of the 40 S RNA (Oker-Blom et al., 1984). It serves as a messenger RNA for the synthesis of RV structural proteins (Oker-Blom et al., 1984). The molecular mechanism that results in the synthesis of the 24 S subgenomic RNA is not clear, but presumably involves a negative-sense RNA intermediate. Sequences of the 24 S subgenomic RNA have been determined from cDNA clones for wild type isolates (M33 and Therien strain) (Clarke et al., 1987; Frey et al., 1986) or vaccine strains (RA 27/3 and HPV 77) (Nakhasi et al., 1989; Zhang et al., 1989). A 95% homology at the nucleotide level is found between three reported RV 24 S RNA sequences whereas little homology was found with that of the alphavirus subgenomic mRNA (Frey and Marr, 1988).

Recently cDNA clones covering the entire RV genome for the Therien (Dominguez et al., 1990) and M33 strains (Yang et al., 1993) have been constructed and sequenced. Sequence data derived from cDNAs reveal that the RV genome is 9756 nucleotides (Therien) or 9764 (M33) in length and has a G/C content of 69.5%, the highest of any RNA virus sequence to date (Dominguez et al., 1990). Alignment of the 40 S sequence between the Therien and M33 strains shows homologies of 97.5% and 96.6% for overall nucleotide and deduced amino acid sequences, respectively (Yang et al., 1993). The RV genome contains two long open reading frames (ORFs),
a 5' proximal ORF (from nucleotide 41 to nucleotide 6385) which encodes the nonstructural proteins and a 3' proximal ORF (from nucleotide 6506 to nucleotide 9694) which encodes the structural proteins (Fig.2) (Dominguez et al., 1990; Yang et al., 1993). Thus, the genomic organization of RV closely resembles that of alphaviruses (Strauss et al., 1984).

1.3.5. Non-structural proteins

The non-structural proteins of RV are encoded by the 5' two-thirds of its genome, and translated as a polyprotein precursor. In RV infected cells, protein species with molecular masses of 200, 150, 87, 75 and 27 kDa, in addition to the structural proteins, have been detected using human convalescent serum (Bowden and Westaway, 1984). Recently, RV-specific proteins with electrophoretic mobilities corresponding to 200, 150, and 90 kDa have been expressed in cells transfected with a recombinant plasmid (pTM3/nsRUB) containing the RV 5' proximal ORF under the control of the T7 polymerase promoter (Marr, et al., 1994). Antibodies raised against bacterial fusion proteins containing regions encoded by the 5' proximal ORF react to the 200, 150 and 90 kDa proteins from the above mentioned cDNA transfected cells as well as RV-infected cells. Mutational analysis indicates that the 150 and 90 kDa proteins are the processing products of the 200 kDa precursor and the order within the ORF is NH2-P150-P90-COOH (Marr et al., 1994, Forng and Frey, unpublished results).

The biological functions of these proteins is not known. Amino acid sequences predicted from cDNA sequences reveal a conserved helicase motif and a replicase motif found among well-studied positive-stranded RNA viruses. In addition, a cysteine protease activity is found to be involved in the processing of the nonstructural protein precursor and an important catalytic role
1.3.6. Expression and processing of structural proteins

RV contains three structural proteins: a capsid protein, C (33 kDa), and two membrane glycoproteins E1 (57 kDa) and E2 (42-47 kDa). In RV infected cells, the structural proteins are translated as a polyprotein precursor, in the order, NH$_2$-C-E2-E1-COOH, with the 24 S subgenomic RNA serving as a template (Fig.3) (Oker-Blom et al., 1984). The polyprotein precursor is subsequently proteolytically processed to yield three individual structural proteins. Unlike SIN and SFV, the RV capsid protein does not possess an autocatalytic serine protease-like activity used to release itself from the polyprotein (Clarke et al., 1987; McDonald et al., 1990). In the absence of microsomes, in vitro translation of the 24 S subgenomic RNA produces a polyprotein precursor of 110 kDa (Oker-Blom et al., 1984; Clarke et al., 1988).

Amino acid sequences predicted from cDNAs reveals that both RV E1 and E2 are type I membrane proteins (Singer et al., 1987) with their N-termini preceeded by stretches of 20 and 23 hydrophobic amino acid residues, respectively (Clarke et al., 1987; Frey and Marr, 1988). These hydrophobic sequences resemble the consensus signal peptides that mediate targeting of nascent polypeptides to the ER membrane and initiate translocation of protein into the ER lumen (reviewed by Wiley, 1986). In vitro and in vivo expression of wild-type and mutant proteins lacking the signal sequences demonstrate that the presence of the signal peptides is required for translocation and processing of the polyprotein precursor (Hobman et al., 1988; Hobman and Gillam, 1989; Marr et al., 1991). Furthermore, mutations at the cleavage sites (von Heijne, 1984) of either the E2 or E1 signal peptide resulted in the accumulation of uncleaved polyprotein
Fig. 2 Topography of the genome RNA of RV. The scale at the top of diagram is in kilobases. Untranslated sequences are denoted by black lines and open reading frames (ORFs) by open boxes. The 5' proximal ORF encodes nonstructural proteins and 3' proximal ORF encodes structural proteins. The boundaries of the individual proteins processed from the precursor translated from each ORF are denoted. Within the nonstructural protein ORF, the location of global amino acid motifs indicative of replicase (R), helicase (H), and cysteine protease activity (P) as well as the small regions of homology between the deduced amino acid sequence of RV and SIV (X motif) is shown. Also shown are: positions of regions of nucleotide homology between RV andalphaviruses, (open circle); subgenomic start site, (closed circle); the 3' terminal stem-and-loop structure, (hatched circle). An expanded topography of the RV structural protein ORF is shown at the bottom of the diagram. Within the ORF, the positioning of the following domains of the structural proteins are shown: the hydrophilic region of C which contains a high concentration of basic amino acids and putatively interacts with the virion RNA; the hydrophobic signal sequences which proceed the N-termini of E2 and E1; the transmembrane sequences of E2 and E1; potential N-linked glycosylation sites (the site marked with a Y is not present in the HPV-77 and M33 strain); a putative region for O-linked glycosylation. Below the diagram are shown the location of domains which contain epitopes recognized by mouse mAbs. (N denotes domains containing epitopes recognized by neutralizing mAbs). (Frey, 1994).
precursor (McDonald et al., 1991; Qiu et al., 1994). Therefore, it is clear that the cleavage of E1 and E2 signal peptides by cellular signalase gives rise to individual RV structural proteins during the processing of polyprotein precursor.

1.3.7. Posttranslational modification of RV structural proteins

1.3.7.1. Capsid protein

The capsid protein of RV is nonglycosylated and associates with the genomic RNA in RV-infected cells to form nucleocapsid. The cDNA sequence indicates that the C protein has a maximal size of 300 amino acid residues with high percentages of arginine and proline residues (Clarke et al., 1987; Frey and Marr, 1988). Capsid protein from virions migrates as a doublet in polyacrylamide gels (Suomalainen et al., 1990; Marr et al., 1991; Maraucher et al., 1991), the differences in molecular weight presumably being due to the alternative sites for translation initiation. Recently it has been shown that capsid protein is phosphorylated, although the extent and function of phosphorylation is unclear (Sanchez and Frey, 1991). After the cleavage of the E2 signal peptide during translocation, the E2 signal peptide is found attached to capsid protein, which helps the capsid protein to become membrane-associated (Suomalainen et al., 1990). The E2-signal sequence-mediated membrane association of the C protein may be important in the transport of the C protein and in nucleocapsid formation.

1.3.7.2. E2 glycoprotein

On an SDS gel, E2 glycoprotein from virion migrates as a diffuse band with molecular weights ranging from 42 to 47 kDa. In the presence of tunicamycin, Vero cells infected with RV and
COS cells transfected with RV cDNAs produce E2 with a molecular weight of 29 to 31 kDa (Oker-Blom et al., 1983; Sanchez and Frey, 1991) and thus the carbohydrate contributes one-third of the molecular mass of E2. Amino acid sequence predicted from E2 cDNAs reveals a protein of 281 residues including three potential N-linked glycosylation sites in M33 (Clarke et al., 1987).
and HPV77 (Zheng et al., 1989) strains as opposed to four in Therien (Vidgren et al., 1987; Frey and Marr, 1988) and RA27/3 (Frey et al., 1986) strains. In addition to N-linked glycans, E2 is known to contain O-linked carbohydrates (Sanchez and Frey, 1991; Lundstrom et al., 1991). Digestion with glycosidases and lectin-binding assays reveal that N-linked glycans on E2 from virions contain high-mannose, hybrid-type and complex-type (Putnam and Therien strains) (Bowden and Westaway, 1985; Sanchez and Frey, 1991) or only complex-type, four branched sugars (M33 strain) (Lundström et al., 1991), with the majority of complex-type terminating in galactose and some fraction having terminal sialic acid (Sanchez and Frey, 1991; Lundstrom et al., 1991). The heterogeneous processing of both N-linked and O-linked glycans on E2 contributes to the diffuse nature of E2 on an SDS gel.

Expression of E2 in vitro and in vivo from cDNA constructs demonstrates that translocation of E2 into the lumen of the rough ER is mediated by a signal peptide residing in the C-terminus of the capsid protein, and this sequence can function externally as well as in its native internal context (Hobman and Gillam, 1989; Marr et al., 1991; Sanchez and Frey, 1991). Following translocation, N-linked glycosylation of E2 takes place. Processing of N-linked glycans on E2 involves at least two stable intermediates, a 39 kDa high mannose-containing precursor and a 42 kDa form bearing some complex-type sugars (Hobman and Gillam, 1989; Hobman, et al., 1990). Although it has been shown that E2 contains O-glycans, the site of O-linked glycosylation and the extent of processing have not been defined. So far the importance of N- and O-linked oligosaccharides on E2 in virion assembly and infectivity is unknown.
1.3.7.3. E1 glycoprotein

E1 is the dominant surface molecule of the RV virion (Ho-Terry and Cohen, 1984; Terry et al., 1988). E1 migrates as a discrete band with apparent molecular weight of 57 kDa. Nonglycosylated E1 synthesized in tunicamycin-treated cells has a molecular weight of 53 kDa (Oker-Blom et al, 1983; Bowden and Westaway, 1984; Sanchez and Frey, 1991). Deduced amino acid sequence shows that E1 is 481 amino acid residues in length with three potential N-linked glycosylation sites (Frey et al., 1986; Clarke, et al., 1987). O-linked oligosaccharides are not detected in E1 (Lundstrom at al., 1991) whereas palmitic acid is incorporated in E1 (Hobman et al., 1990). There is a stretch of seven amino acids including five arginine residues (R-R-A-C-R-R-R-R) before the putative signal peptide sequence of E1 and after the putative transmembrane anchor domain of E2 that may contain basic amino acid cleavage sites for endoproteases. Since the C-terminal amino acid sequence of E2 has not been determined, it is not known whether other proteolytic cleavages take place during the processing of the E2E1 precursor polyprotein at the C-terminus of E2, besides the cleavage of the E1 signal peptide by host signal peptidase. A recent mutational study of this region shows that the cleavage of the E2E1 polyprotein precursor is impaired when the signal peptide cleavage site alone, or both arginine clusters are altered, whereas partial cleavage is observed in the mutants in which one of the two arginine clusters is modified (Qiu et al., 1994). These data indicate that the arginine clusters do not function as a basic protease cleavage site, rather, they contribute to maintaining the proper configuration of that region for access by cellular signal peptidase.
1.3.7.4. Conformation of structural proteins

Capsid protein forms a noncovalently bound dimer soon after translation in RV-infected cells as well as in cells infected with a vaccinia recombinant virus expressing C protein (Baron and Forsell, 1991). However, covalently linked C dimers are detected only in RV-infected cells but not in vaccinia recombinant-infected cells (Baron and Forsell, 1991). Similarly, disulfide-bound E1-E1 homodimers and E1-E2 heterodimers are routinely observed when rubella virions are subjected to non-reducing SDS-PAGE (Waxham and Wolinsky, 1983; Dorsett et al., 1985), whereas such glycoprotein complexes are not detected when E1 and E2 are expressed from cDNA (unpublished results). It is possible that the formation of intermolecular disulfide bonds of E1 and E2 occur after virions are released from the infected cells and exposed to a relatively oxidative environment in the medium. Besides intermolecular disulfide bonds, intramolecular disulfide bonding is found in E1 and E2 which is important to the maintenance of proper conformation for antibody binding (Green and Dorsett, 1986; Wolinsky et al., 1991), protein stability, hemagglutination activity and infectivity (Ho-Terry and Cohen, 1981; Katow and Sugiura, 1988).

Intracellularly, E1 and E2 form noncovalently associated heterodimers in RV-infected cells as well as when they are expressed from cloned cDNAs (Baron and Forsell, 1991; Hobman et al., 1993). The association of E1 and E2 increases the intracellular transport rate of E2 (Hobman et al., 1990) and releases E1 from retention in a post-ER, pre-Golgi compartment (Hobman et al., 1993).
1.3.8. Intracellular localization of RV structural proteins

In an indirect immunofluorescence study, RV glycoproteins E1 and E2 were shown to be concentrated in the juxtanuclear region of both RV-infected cells and cDNA transfected cells expressing all three structural proteins of RV. This region represents a reticular structure which may span from the ER to the Golgi stacks. Low level cell surface expression of E1 and E2 is also observed (Hobman et al., 1990). The capsid protein is also localized in the Golgi-like area, presumably due to its membrane association as well as the interaction with E1 and/or E2 (Hobman et al., 1990; Baron et al., 1992). However, when each protein is expressed separately from cloned cDNA, a different intracellular distribution pattern is observed. The capsid protein is found in a reticular structure extending throughout the cells (Baron et al., 1992). E2 is in the ER, the Golgi cisternae and the cell surface, whereas E1 is retained in a pre-Golgi structure (Hobman et al., 1990). Fine mapping of the E1 and E2 intracellular localization using immunogold labelling reveals that E1, when expressed alone, is arrested in a novel post-ER, pre-Golgi compartment near the exit site of the ER (Hobman et al., 1992). Although the co-expression of E1 and E2 releases such retention, they are targeted to the Golgi complex, and are not efficiently transported to the cell surface (Hobman et al., 1993).

1.3.9. Biological function of RV structural proteins

The major biological activities associated with the RV virion (structural proteins) are hemagglutination (HA) (Schmidt et al., 1968) and low-pH induced cell-cell fusion of infected cells (Vaananen et al., 1980). There is evidence linking both HA and fusion activities to RV E1. Trypsin treatment of virions under conditions which digest E1 with minimum damage to E2
shows that such digestion results in the loss of the HA activity (Ho-Terry and Cohen, 1981). Murine mAbs that exhibit hemagglutination inhibition (HAI) activity, are all anti-E1 but not anti-E2 (Waxham and Wolinsky, 1983; Green and Dorsett, 1986; Chaye et al., 1992). More direct evidence is that E1 but not E2 expressed via vaccinia virus recombinants can induce the production of hemagglutination inhibitory antibodies (Gillam, unpublished result).

Brief exposure of RV infected cells to a pH of 6.0 or lower results in syncytium formation, presumably mediated by the RV glycoproteins expressed at the plasma membrane (Katow and Sugira, 1988). RV virions also induce fusion of erythrocytes (Vaaninen et al., 1980) and the virions gain the ability to bind liposomes (Katow and Sugiura, 1988) after incubation in acidic media. The basis of the fusogenic activity in RV is not well defined but is thought to reside in E1.

1.3.10. Immune responses to RV infection

Natural RV infection or RV vaccination leaves a long-lasting immunity, which is attributed to circulating antibodies. The initial response following infection or vaccination is a transient IgM response and in most cases is E1 specific (Partanen et al., 1985). Although other immunoglobulin classes (IgE, IgA) are stimulated subsequently, IgG production is the dominant serological response. Persisting IgG antibodies are directed to all three structural proteins of RV, although the predominant reactivity is against E1 (Katow and Sugiura 1985; Zhang et al., 1992; Chaye et al., 1992), indicating an important role of E1 in inducing a protective immunity against RV infection.

IgG antibodies to RV may have HAI and viral neutralizing (VN) properties (Green and
Dorsett, 1986; Waxham and Wolinsky, 1985). There is a good correlation between the levels of IgG to RV, and classically measured HAI titres and neutralizing antibody titres in seropositive sera (Stokes et al., 1969). It is assumed that these responses play a positive role in viral clearance and protection (Waxham and Wolinsky, 1985a). Circulating immune complexes containing RV specific antibody and antigen are frequently found after RV infections (Ziola et al., 1983) but in most cases their presence has not been associated with any of the complications following RV infection or vaccination (Singh et al., 1986).

Much less is known about the importance of cellular responses to RV infection. RV-specific cellular responses have been demonstrated using lymphocyte proliferation assays and lymphocyte mediated cytotoxicity assays (Buimovici-Klein and Cooper, 1985; Vesikari and Buimovici-Klein, 1974; Ilonen and Salmi, 1986). Cell-mediated cytotoxicity has been implicated in the pathogenicity of RV infection (Martin et al., 1989). In these studies, intact RV was used as the antigen for the analysis of proliferation responses. Only recently, Chaye et al. (1992) demonstrated antigen-specific lymphocyte proliferative responses in peripheral blood lymphocytes using an in vitro proliferative assay with vaccinia recombinants expressing individual RV structural proteins. In human populations, each individual exhibits different responses to E1, E2 and C; however, E1 is the dominant antigen to which the majority of subjects develop lymphocyte proliferative responses (Chaye et al., 1992).

Proliferative responses to purified, intact RV are major histocompatibility antigen (HLA) restricted (Ilonen and Salmi, 1986). Similarly, Ou et al. (1992a,b,c) isolated T-cell clones against E2 glycoprotein and C protein from RV seropositive individuals and found that HLA restrictions were associated with HLA DR7 for E2 epitopes, and HLA DR4 for C epitopes.
1.3.11. Immunological determinants on RV structural proteins

To characterize the antigenic determinant on RV structural proteins, panels of murine mAbs have been generated and biological activities of these antibodies have been analyzed. These panels are made up primarily by E1-specific antibodies with a rare number of antibodies recognizing E2 or C (Waxham and Wolinsky, 1985; Green and Dorsett, 1986; Chaye et al., 1992). In 1985, Waxham and Wolinsky (1985) mapped HA and VN activities to the E1 glycoprotein. Since this study, much effort has been focused on delineating functional epitopes on E1 (Terry et al., 1988, 1989; Lozzi et al., 1990; Wolinsky et al., 1991). Numerous methods to localize HA or VN epitopes have been utilized including recombinant DNA technology (Terry et al., 1989; deMazancourt and Perricaudet, 1989; Wolinsky et al., 1991), peptide analysis (Lozzi et al., 1990; Terry et al., 1988; Mitchell et al., 1992) and competitive binding assays with mAbs (Waxham and Wolinsky, 1985a). Six independent epitopes have been identified which are thought to be important for viral infectivity and HA (Green and Dorsett, 1986; Waxham and Wolinsky, 1985a). Three non-overlapping linear epitopes that react with mAbs having HAI and VN activities have been localized to E1 residues 245 to 285 (Terry et al., 1988). Wolinsky et al. (1991) and Chaye et al. (1991) separately mapped a region between residues 202 to 283 of E1 which consists of overlapping epitopes recognized by mAbs with VN, HAI or VN and weak HAI activities (Wolinsky et al., 1991; Chaye et al., 1992). Using a set of nested synthetic peptides, these two groups subsequently narrowed the epitope to a region between residues 213 and 239, or between residues 214 and 240, respectively (Chaye et al., 1992; Wolinsky et al., 1993). Contrary to those results with E1, B cell-epitope mapping on E2 and C has been less informative, due to the lower number of mAbs available. Only one of the E2-specific mAbs has been found to possess VN
activity (Green and Dorsett, 1986). It was reported recently that an anti-C mAb recognizes a 52 kDa β-cell antigen, implying that the C protein may be involved in molecular mimicry leading to initiation of an immunopathological process (Karounos et al., 1993).

Protective immunity to viral infection requires activation of helper T cells specific for viral antigens. Ou et al. (1992a, b, c, 1993) identified T-cell epitopes on RV structural proteins by screening a nested set of overlapping synthetic peptides with peripheral blood lymphocytes from immune donors and subsequently with T-cell lines/clones derived from the peripheral blood lymphocytes of immune donors. They identified regions between residues 358 and 377 of E1, between residues 54 and 74 of E2, and between residues 255 and 280 of C, as the relatively immunodominant T-cell epitopes (Ou et al., 1992a,b,c, 1993). Mitchell et al. (1993), applying essentially the same methodology, identified immunoreactive regions on E1 and E2 recognized by T-cells of normal healthy individuals. McCarthy et al. (1993) took a different approach, using sets of comparatively short overlapping synthetic peptides containing predicted T-cell epitope motifs of RV structural proteins within the region bearing linear B-cell epitopes defined by RV-specific mAbs (C₁ to C₂₉, C₆₄ to C₉₇, E₂₃₁ to E₂₁₀₅ and E₁₂₀₂ to E₁₂₈₃). With one exception, all of the synthetic peptides were able to stimulate varied but individually specific lymphoproliferative responses in peripheral blood mononuclear cells from 25 to 50% of a population of normal, RV-immune donors with diverse HLA backgrounds (McCarthy et al., 1993). These studies indicate that further fine-mapping of T-cell determinants among a larger human population with HLA diversity is necessary for future construction of an effective synthetic peptide vaccine for RV.
1.3.12. Project rationale and thesis objectives

Viruses utilize the host cell machinery for the synthesis and processing of viral proteins. Viral proteins undergo a series of structural modulations during transport from the site of synthesis to the site at which they are incorporated into virions, and become functionally competent. Post-translational modifications are a major focus of studies on structure/function relationship of proteins, and among them, glycosylation has been studied exhaustively. RV contains two membrane glycoproteins E1 and E2. In recent years, although studies on a) the structure of carbohydrates on E1 and E2 (Sanchez and Frey, 1991; Lundstrom et al., 1991), b) the intracellular transport and processing of E1 and E2 (Hobman and Gillam, 1989; Hobman et al., 1990; Sanchez and Frey, 1991; Baron and Forsell, 1991; Marr et al., 1991; Baron et al., 1992) and c) the analysis of immunological determinants on E1 and E2 (Wolinsky et al., 1991; Chaye et al., 1992) have greatly strengthened our knowledge about RV, little is known about the functional role of N-linked glycosylation on RV E1 and E2.

In this study, the importance of N-linked oligosaccharides on RV E1 and E2 has been investigated with respect to its biological functions during replication and infection. The approaches taken involve a combination of recombinant DNA technology and mammalian cell expression. The thesis describes two lines of experiments. The first line of experiments is directed at the cell biology aspects of E1 and E2. Studies were initiated to define the role of N-linked glycosylation on processing and transport of E2, and these experiments were extended to investigate the correlation between the sorting of the E1 and E2 glycoproteins and virus assembly using two protein transport inhibitors, brefeldin A and monensin. The second line of experiments are focused on vaccine development. In view of the fact that the immunoreactivity of RV E1
glycoprotein is very dependent on its native conformation, the influence of N-linked glycosylation of E1 on its antigenicity and immunogenicity was analyzed. The outcome of these experiments led to the expression and characterization of the virus-like particles containing RV structural proteins, and studies of their immunological properties. The potential application of these virus-like particles as an antigen sources for serodiagnostic assays and vaccine development will be discussed.
2. MATERIALS AND METHODS

2.1. MATERIALS and SUPPLIES

DNA modifying enzymes and restriction endonucleases were purchased from Bethesda Research Laboratories (BRL), Promega, New England Biolabs, Boehringer Mannheim, Pharmacia and United States Biochemical Corporation. All enzymes were used as specified by the manufacturer unless indicated otherwise. L-[\textsuperscript{35}S]-methionine (600-800 Ci/m mole) was from Du Pont Inc. Tissue culture reagents were from Gibco (Gaithersburg, MD) or Sigma (St. Louis, IL). Brefeldin A was purchased from Boehringer Mannheim. Tunicamycin and monensin were products of Sigma. GENECLEAN (BIO 101) was obtained from Promega. Human polyclonal anti-rubella serum was provided by Dr. A. Tingle (B.C. Children's Hospital, Vancouver, B.C.). Mouse monoclonal antibodies against RV E1 were generated in this lab. Mouse monoclonal antibodies against RV E2 and capsid protein were generously provided by Dr. J. Safford (Abbott Laboratories, Chicago, IL) or Dr. J. Wolinsky (University of Texas, Houston, TX). Fluorescein (FITC)-conjugated goat anti-mouse or anti-human IgGs were from Kirkegaard & Perry Laboratory. Rhodamine (TRICT)-conjugated goat anti-mouse or anti-human antibodies, and lectins were from Zymed. A TRICT-conjugated rabbit anti-Golgi protein serum was prepared in the laboratory of Dr. F. Tufaro (University of British Columbia, Vancouver, B.C.). COS, CV-1, tk'143, BHK, Vero and RK cells, rubella virus M33 strain and vaccinia virus WR strain were obtained from the American Type Culture Collection.
2.2. METHODS

2.2.1. Propagation of bacterial strains

E.coli strains DH5α from BRL were used for the propagation of recombinant clones. DH5α cells containing recombinant plasmids were grown in LB medium (1% tryptone; 0.5% yeast extract; 1% NaCl) containing 100 ug/ml ampicillin (AP) for selection of antibiotic resistance. For long term storage the bacterial strain was stored in 15% glycerol at -70°C.

2.2.2. Preparation of competent cells and transformation

Competent cells were prepared using a method described in Promega technical bulletin 018. Briefly, E.coli cells were grown in 20 ml of LB medium until the absorbance at 600 nm reached 0.15-0.3. Cells were centrifuged at 5000 rpm in a Sorvall SS34 rotor at 4°C for 5 minutes, and the supernatant was discarded. The bacterial pellet was resuspended in 10 ml of cold solution A (10 mM 3-[N-morpholino] propanesulfonic acid (MOPS) (pH 7.0); 10 mM RbCl), and centrifuged as above. Cells were then resuspended in 10 ml of cold solution B (10 mM MOPS (pH 6.5); 10 mM RbCl; 50 mM CaCl₂) and incubated on ice for 30 minutes. After pelleting the cells as above, cell pellets were resuspended in 1 ml of solution B plus 15% glycerol, and quick frozen in 0.2 ml aliquots in dry ice-ethanol and stored at -70°C.

For plasmid transformation, 0.2 ml of competent cells were incubated on ice with 10-50 ng of plasmid DNA for 30 minutes. After a two minute heat shock at 42°C, 1 ml of LB medium was added to the transformation mixture and the cells were allowed to recover at 37°C for 45 minutes before plating onto selective media.
2.2.3. DNA preparation and handling

2.2.3.1. Mini-prep plasmid isolation

Colonies containing plasmids were picked into 3-5 ml of LB containing 100 µg AP per ml and the bacteria were grown to saturation. Bacterial cells from 1.5 ml culture were pelleted for one minute in a microfuge. The pellet was resuspended in 100 µl of 50 mM glucose; 10 mM EDTA; 25 mM Tris-HCl (pH 8.0) and lysed by the addition of 200 µl of 0.2 N NaOH/1% SDS for 5 minutes at 0°C. Chromosomal DNA and proteins were precipitated by incubating the lysis mixture with 150 µl of cold potassium acetate (3 M K+, 5 M CH₃COO, pH 4.8) at 0°C for 5 minutes, followed by centrifuging in a microfuge for 5 minutes at 4°C. The supernatant was extracted with an equal volume of phenol:chloroform (1:1), and the DNA precipitated with two volumes of ethanol at room temperature (RT) for 5 minutes. Plasmid DNA was recovered by centrifugation in a microfuge for 5 minutes at RT, washed in 70% ethanol, dried in a Speed Vac Concentrator, and resuspended in 50 µl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) containing 20 µg/ml RNase A. Aliquots were used for restriction analysis or subcloning. This method is essentially that described by Maniatis et al. (1982).

2.2.3.2. Large scale plasmid DNA preparations

The protocol is a procedure obtained from Promega technical bulletin 009 (developed by Dr. P. Krieg and Dr. D. Melton of Harvard University) with modifications. Cells grown in selective media overnight in 100 ml cultures were pelleted by centrifugation at 5000 rpm in a Sorvall GSA rotor at 4°C for 5 minutes. The supernatant was discarded and each pellet was resuspended in 3 ml of 50 mM glucose; 10 mM EDTA; 25 mM Tris-HCl (pH 8.0) containing 20 mg/ml
lysozyme followed by 20 minute incubation on ice. Cells were lysed by the addition of 6 ml of 0.2 N NaOH/1% SDS and incubation on ice for 10 minutes. Chromosomal DNA and proteins were precipitated with 4 ml of cold potassium acetate solution (see mini-prep procedure) on ice for 20 minutes, followed by centrifugation at 15,000 rpm in a Sorvall SS34 rotor at 4°C for 15 minutes. RNase A (100 ug) was added to the cleared lysate followed by incubation at 37°C for 20 minutes. The lysate was extracted twice with equal volumes of phenol:chloroform, and the nucleic acids were precipitated with one volume of isopropanol at RT for 5 minutes. Remaining nucleic acids were recovered by centrifuging at 15,000 rpm for 10 minutes at 4°C in a SS34 rotor. The pellet was dried, and dissolved in 1.60 ml of sterile water. The solution was transferred to siliconized Corex tubes and DNA was selectively precipitated by the addition of 0.4 ml of 4 M NaCl and 2.0 ml 13% polyethylene glycol (PEG, MW 8,000), mixing and incubation on ice for 60 minutes. The plasmid DNA was pelleted at 10,000 rpm for 10 minutes at 4°C in a SS34 rotor, washed with 70% ethanol, dried and dissolved in TE.

2.2.3.3. Restriction endonuclease digestions and DNA modification

All restriction digestion reactions were performed according to assay conditions specified by the suppliers.

DNA fragments were ligated using T4 DNA ligase in 50 mM Tris-HCl, pH7.6; 10 mM MgCl₂; 1 mM ATP; 1 mM DTT; 5% (w/v) polyethylene glycol for 2 hours at RT, except for blunt-ended fragments which were ligated overnight. Reactions were diluted five-fold with TE prior to transformation (Maniatis et al., 1982).

DNA fragments with 5' overhangs were blunt-ended with E.coli DNA polymerase I Klenow
enzyme in 50 mM Tris-HCl, pH 7.2; 10 mM MgSO₄; 10 mM DTT; 50 mM BSA; 80 μM dNTP's for 30 minutes at RT. The enzyme was inactivated by heating at 70°C for 5 minutes (Maniatis et al., 1982).

Fragments with 3' protrusions were converted to flush ends using T4 DNA polymerase in 33 mM Tris-acetate, pH 7.9; 666 mM potassium acetate; 10 mM magnesium acetate; 0.5 mM DTT; 100 mg/ml BSA; for 5 minutes at 37°C. Reactions were adjusted to 25 mM EDTA, and the DNA purified by phenol:chloroform extraction and ethanol precipitation (Maniatis et al., 1982), or using GENECLEAN (BIO 101).

Removal of terminal 5' phosphates from DNA fragments with 5' overhangs was done using calf intestinal alkaline phosphatase (CIP) in 50 mM Tris-HCl, pH 9.0; 1 mM MgCl₂; 0.1 mM ZnCl₂; 1 mM spermidine for two successive 30 minute incubation periods of 15 minute at 37°C and 15 minutes at 56°C. CIP reactions were terminated by addition of 0.3% SDS and phenol:chloroform extraction followed by ethanol precipitation (Maniatis et al., 1982).

Purification of DNA fragments from agarose gels or enzyme reaction mixtures was routinely done using GENECLEAN. Desired fragments were excised from ethidium bromide stained TAE agarose gels (see 2.2.12.1) and the gel matrix was solubilized in 2-3 volumes of saturated sodium iodide at 55°C. DNA was removed from the agarose solutions by vortexing the mixture with a suspension of glassmilk, and a brief spin in a microfuge. Contaminants were washed away from the glass bound DNA by three successive washes with cold NaCl/ethanol/water solution. The DNA was eluted from the glass beads with TE or water by incubating at 55°C for 3 minutes.
2.2.4. Expression vectors

2.2.4.1. pCMV5

For transient expression of RV cDNA in COS cells, pCMV5 (Andersson et al., 1990) was used (Fig. 4a). This vector directs transcription by the human cytomegalovirus major immediate early gene promoter and provides polyadenylation signal from the human grown hormone gene at the 3' terminus of the inserted sequence. pCMV5 contains the SV40 origin of replication allowing replication in COS cells as well as a prokaryotic origin of replication and AP resistance gene for growth and selection in *E. coli*.

2.2.4.2. pGS20

For construction of vaccinia recombinants, vector pGS20 (Fig. 4b) (Mackett et al., 1985) was used. This vector contains the vaccinia virus immediately early gene promoter p7.5, flanked by sequences from the vaccinia virus thymidine kinase gene. The AP resistance gene and prokaryotic replication origin allow propagation of the recombinant plasmid in *E. coli*.

2.2.4.3. pNUT

Vector pNUT (Fig. 4c) (Palmiter et al., 1987) was used to construct stable transformed BHK cells. RV cDNAs were cloned into this vector at the Sma I site which is flanked by the mouse metallothionein gene (mMT-1) promoter and the 3' polyA sequences of the human growth hormone (hGH). The presence of the dihydroxyfolate reductase (DHFR) cDNA permits the selection of transfected BHK cells in the presence of a high concentration of methotrexate. Sequence from the human hepatitis B virus 3' end mediates insertion of adjacent DNA sequences
into the chromosome of host cells (Nagaya et al., 1987). The pUC18 backbone and SV40 origin facilitate replication of the plasmid in *E.coli* and mammalian cells, respectively.

### 2.2.5. DNA-mediated transfection

#### 2.2.5.1. Transfection of COS cells

COS cells were transfected with plasmid DNA using a method described by Adam and Rose (1985). Subconfluent monolayers of cells grown in Dulbecco modified Eagle medium (DMEM) plus 5% fetal calf serum (FCS) were washed twice with Tris-saline (25 mM Tris-HCl (pH 7.4), 140 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.9 mM Na₂HPO₄). Cells were incubated with DEAE-dextran (Mr=5 X 10⁵; 500 μg/ml) and plasmid DNA (4 μg/ml) in Tris-saline at 37°C for 30 minutes. The DNA solution was then removed and replaced with DMEM plus 40 μM chloroquine for 3 hours at 37°C. After removal of chloroquine solution, the cells were shocked with 10% dimethylsulfoxide/DMEM for 3 minutes at RT. Finally, the monolayer was washed three times with Tris-saline and incubated at 37°C for 40 hours in DMEM plus 5% calf serum. The expression of RV proteins was analyzed using metabolic labelling or immunoblotting (see below).

#### 2.2.5.2. Calcium-phosphate mediated DNA transfection

Transfection of CV1 cells and BHK cells with plasmid DNAs were according to Gorman et al., (1982). CaPO₄/DNA mixture was prepared by combining 10-25 μg plasmid DNA in 219 μl of ddH₂O, 31 μl of 2M CaCl₂ and 250 μl of 2xHBSP (1.5 mM Na₂HPO₄; 10 mM KCl; 280 mM NaCl; 12 mM glucose; 50 mM HEPES, pH 7.0). The mixture was allowed to stand for 30...
Fig. 4 Schematic representation of mammalian cell expression vectors used in this study. 

a. pCMV5. The vector backbone is pTZ18R (Pharmacia) and contains a bacteriophage fl ampicillin-resistance gene (Amp'). The CMV region consists of a promotor-regulatory region of the human cytomegalovirus major immediate early gene. 

b. pGS20. The vector contains the promotor for an early gene coding for a 7.5 kDa polypeptide and is placed upstream from the unique restriction endonuclease Sma I site, flanked by vaccinia thymidine kinase (TK) DNA (thick line).

c. Recombinant plasmid from pNUT vector. The backbone for this vector is pUC18. The essential features of this vector are: DNA sequences taken from the 3' termini of human hepatitis B virus genome (HBV 3'); a promotor region from mouse metallothionein I gene (mMT-1); and sequence for dihydrofolate reductase (DHFR). RV cDNAs were inserted into the Sma I site between the mMT-1 and hGH 3' sequences.
minutes at RT prior to adding to the medium of cultured cells. Cells were incubated for different periods of time before removing the DNA mixture. The time of incubation depended on the cell type and nature of experiment.

2.2.6. Construction of vaccinia recombinants

Vaccinia virus recombinants expressing RV E1 glycosylation mutant proteins were constructed following a standard procedure as described by Mackett et al., (1985).

2.2.6.1. Infection/transfection procedure

Confluent monolayers of CV1 cells in Minimal Essential Medium (MEM) were infected with purified vaccinia virus (WR strain) at a ratio of 0.05 p.f.u./cell. Inoculum was removed at 2 hours post infection (h.p.i.). Cells were washed twice with serum-free medium and 0.5 ml of DNA suspension (CaPO₄/DNA) (see 2.2.5.2.) was added to the cells and the cells were incubated for 30 minutes at RT prior to the addition of MEM/5% FCS. Cells were scraped into the medium at 48 h.p.i. and viruses were released by three cycles of freeze-thawing.

2.2.6.2. Selection of recombinants

One-fifth of the released viruses were layered onto monolayers of human tk⁻ 143 cells (gift from F. Graham, McMaster University) and incubated for 1 hour at 37°C. The inoculum was removed and cells were incubated with Eagles medium containing 5% FCS and 25 μg/ml 5-bromodeoxyuridine (B UdR). Progeny virus was harvested at 48 h.p.i., by scraping cells into medium and then three rounds of freeze-thawing.
2.2.6.3. Plaque purification and virus titration

Monolayers of CV1 cells were infected with 0.5 ml of a ten fold serial dilution of virus and incubated at 37°C for one hour, with occasional shaking. The inocula were removed, cells were washed with MEM once and overlaid with MEM containing 5% FCS and 1% noble agar. Cells were stained at 36 h.p.i. with 1% agarose containing 0.1% neutral red. Clear virus plaques were visualized after incubation for 2-3 hours. Plaques were counted and virus infectivity was calculated as plaque forming units/ml (pfu/ml). Well isolated plaques were picked into a Pasteur pipette and virus in agarose plugs were eluted into 0.5 ml MEM and stored at -70°C.

2.2.6.4. Large-scale virus purification

Monolayers of CV-1 cells (in 175 cm² flask) were infected with wild type or recombinant vaccinia viruses at a multiplity of infection (MOI) of 5 and incubated for 48 hours. Cells were harvested and resuspended in 10 mM Tris-HCl, pH 9.0 and homogenized. The nuclear pellet was removed after centrifugation at 750xg for 5 minutes at 4°C. Trypsin (0.25 mg/ml) was added to the supernatant and incubated for 30 minutes at 37°C. The supernatant was then layered on top of an equal volume of 36% sucrose in 10 mM Tris-HCl pH 9.0 and centrifuged at 13,500 rpm in a Beckman SW27 rotor for 80 minutes at 4°C. The pellet was resuspended in 2 ml of 1 mM Tris-HCl pH 9.0 and layered onto continuous sucrose gradients (15-40% in 1 mM Tris-HCl, pH 9.0). The centrifugation was carried out at 4°C, 12,000 rpm for 45 minutes. Banded virus was collected with a syringe through the side of the tube and stored at -70°C.
2.2.7. Metabolic labelling

(i). COS cells. Labelling of COS cells was performed according to Hobman and Gillam (1989). Briefly, 40 hours post-transfection, transfected cells (in 35 mm dishes) were washed once and incubated with methionine-deficient DMEM for 30 minutes prior to the addition of 0.5 ml methionine-deficient DMEM containing 100 μCi [³⁵S]-methionine (Du Pont) and 5% FCS dialyzed against phosphate-buffered saline (PBS). Incubation with [³⁵S]-methionine-containing medium was for 30 minutes. Some cells were further incubated with a chase medium containing 2 mM unlabelled methionine for various periods of time. Cells were washed with cold Tris-saline and lysed with 500 μl of RIPA buffer (1% Triton X-100; 10 mM EDTA; 50 mM Tris-HCl, pH 7.5; 1% sodium deoxycholate; 0.15 M NaCl; 0.1% SDS). After keeping on ice for 5 minutes, lysates were scraped off the plates and cleared of nuclei and debris by centrifugation at 4°C for 5 minutes at 13,000 rpm. The supernatants were subjected to immunoprecipitation.

(ii). Vero cells. Labelling of RV virions released from RV-infected Vero cells was carried out as described by Clarke et al. (1987). At 24 h.p.i., infected cells (in 60 mm dishes) were incubated with methionine-deficient medium for 30 minutes and labeled with 100 μCi [³⁵S]-methionine for 1 hour. Cells were washed with and incubated in MEM with 2.5% FCS for various periods of time. Medium samples were collected and an equal volume of 20% PEG (MW 8,000) in 2.5 M NaCl were added. RV particles were precipitated by centrifugation at 4°C for 10 minutes at 14,000 rpm in an Eppendorf centrifuge after incubation on ice for 1 hour. The virus pellets were resuspended in RIPA buffer and RV-specific proteins were immunoprecipitated with human anti-RV serum and subjected to SDS-PAGE and autoradiography.
2.2.8. Immunoprecipitation

Immunoprecipitation of RV structural proteins from cell lysates was performed according to Hobman and Gillam (1989). Human polyclonal anti-rubella serum, mouse serum or fluid ascites were preincubated with Protein A-Sepharose (Pharmacia) for at least 4 hours at 4°C in binding buffer (100 mM Tris-HCl (pH 7.4); 400 mM NaCl) with constant mixing. The antibody-coated beads were washed twice with binding buffer, and once in lysate buffer (25 mM Tris-HCl (pH 7.4); 100 mM NaCl; 1 mM EDTA; 1% Nonidet P-40). Cells lysates or harvested media were added and mixed with the antibody-coated beads for over 8 hours at 4°C with constant rotation. Beads were washed once with lysate buffer, twice with wash buffer (25 mM triethanolamine; 172 mM NaCl; 1% SDS; 1 mM EDTA), three times with 10 mM Tris-HCl (pH 7.4), and once with water. Antigen-antibody complexes were dissociated from the Protein A-Sepharose by boiling in 1 X SDS dissociation buffer (see below) for 5 minutes, vortexing and pelleting the beads by centrifugation. Supernatants were collected and used for further analysis.

2.2.9. Endoglycosidase digestion

The conditions for endoglycosidase digestion were essentially those described by the manufacturer. Digestion with endoglycosidase H (endo H, Boehringer Mannheim) was carried out in 100 mM sodium citrate buffer (pH 5.5) containing 0.15% SDS. Digestion with O-glycosidase, endoglycosidase F/N-glycosidase F (endo F/PNGase F) and neuraminidase (all from Boehringer Mannheim) were performed in a buffer containing 20 mM sodium phosphate pH 7.0, 10 mM n-octylglucoside, 0.1% SDS. Immunoprecipitates to be digested with N-glycanase were adjusted to 100 mM sodium phosphate (pH 8.6), 1% Nonidet P-40, 100 mM EDTA, 0.5% β-
mercaptoethanol, 0.1% SDS. All incubations were normally for 8 hours.

2.2.10. Immunoblotting

RV antigens were separated by SDS-PAGE and transferred to nitrocellulose filters using a Bio-Rad Trans-Blot apparatus for 60 minutes at 240 mA in 25 mM Tris-HCl; 192 mM Glycine (pH 8.3); 20% methanol. The non-binding sites on filters were blocked by incubating for 30 minutes to overnight in TBS (25 mM Tris-HCl, pH 7.4; 150 mM NaCl) containing 4% powdered skimmed milk. Membranes were then incubated with human anti-RV serum or monoclonal antibodies (at appropriate dilutions) for 1 hour, washed with TBS/0.3% Tween-20 and treated with goat anti-human or goat anti-mouse IgG conjugated to alkaline phosphatase (BRL) for 1 hour. Blots were washed as above and developed with NBT (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indoyl phosphate). All incubations were done at RT.

2.2.11. Indirect immunofluorescence

Transfected COS cells grown on polylysine-coated 9 mm glass coverslips were washed three times with PBS, and fixed for 20 minutes at RT in 2% formaldehyde/PBS, followed by washing with PBS. Some cells were permeabilized with 0.1%NP-40/PBS for 30 minutes prior to blocking with 1% BSA/PBS. BSA/PBS was substituted for PBS in all dilutions and washings after this step. Coverslips were overlaid with diluted human serum (1:200) or murine monoclonal antibodies (1:100), incubated for 60 minutes at RT, and washed. Incubation with secondary antibodies, fluorescein-conjugated goat anti-human or anti-mouse IgG (diluted 1:100) was for 60 minutes. For double-labelling using lectin-conjugates, permeabilized cells were incubated with
wheat germ agglutinin-rhodamine conjugated (WGA-TRICT) to visualize Golgi and post-Golgi structures or concanavalin A-rhodamine conjugated (Con A-TRICT) for ER staining at 10-15 μg/ml for 30 minutes at RT prior to blocking with BSA.

2.2.12. Electrophoresis

2.2.12.1. Separation of DNA fragment

The buffers used in agarose gel electrophoresis were 1XTAE (40 mM Tris-acetate, pH 8.0; 1 mM EDTA) and 1XTBE (89 mM Tris; 89 mM boric acid; 2 mM EDTA; pH 8.0) for separation of small fragments. The gel concentration varied from 1% to 2% agarose with 1 μg/ml ethidium bromide for visualization. DNA samples were diluted to 8% sucrose; 20 mM EDTA (pH 8.0); 0.05% bromophenol blue; 0.05% xylene cyanol and separated by electrophoresis on 10 cm submarine horizontal agarose gels at 75 volt.

2.2.12.2. Separation of protein

Proteins were separated using a discontinuous gel system described by Laemmli (1970). Samples were adjusted to 62.5 mM Tris-HCl (pH 6.8); 10% glycerol; 2% SDS; 2% β-mercaptoethanol and denatured at 95°C for 3 minutes. Stacking gels consisted of 4% polyacrylamide, and separating gels contained either 10% or 11% polyacrylamide. Solutions used to prepare these gels are described in 2.2.12.3 (below). Gels were run at constant voltage of 115-125 volts until the markers have run to the desired position. The stacking gel was trimmed away, and the proteins were either fixed in 10% acetic acid for 15 minutes for fluorography or transferred to nitrocellulose membrane for immunoblot analysis. Fixed gels were immersed in the fluorographic
agent Amplify (Amersham) for 15 minutes, dried under vacuum and exposed to X-ray film at -70°C.

2.2.12.3. Solutions used for electrophoresis:

5X Stacking gel buffer: 0.625 M Tris-HCl (pH 6.8), 0.5% SDS

5X Separating gel buffer: 1.875 M Tris-HCl (pH 8.8), 0.5% SDS

5X Gel running buffer: 0.125 M Tris-HCl; 0.96 M glycine, 0.5% SDS (pH 8.3)

Polyacrylamide Stock: 30% acrylamide, 0.8% N’N’-bis methylene acrylamide

Gels were polymerized by adding ammonium persulfate to 0.05% and TEMED (N’N’N’N’-Tetramethylenediamine) to 0.1%.

2.2.13. RV propagation, purification and titration

2.2.13.1. Virus propagation

Vero cells were normally used to grow RV M33 strain in this study. Subconfluent cells (70%) were infected with RV at a MOI of 5-10 at 37°C for 2 hours. The inoculum was removed and cells were incubated with MEM with 2.5% FCS after washing once with the same medium. The medium was collected at intervals of 24 hours starting at 48 h.p.i., and replaced with fresh medium after each harvesting, till 96 h.p.i. Cell debris were cleared by centrifugation at 3,000 rpm for 5 minutes and virus particles were harvested from the medium by centrifugation at 27,000 rpm for 2 hours. Pelleted virus was suspended in PBS and stored at -70°C.
2.2.13.2. Purification of RV or virus-like particles using sucrose density gradients

Pelleted RV or virus-like particles from 35 ml tissue culture supernatant were suspended in 0.35 ml TNG buffer (50 mM Tris, pH 7.5; 100 mM NaCl; 200 mM glycine) and applied onto the top of a 12 ml-sucrose gradient of 20-50% sucrose in TNG. Centrifugation was carried out using a Beckman SW41 rotor at 90,000xg for 16 hours at 15°C. Fractions (~ 0.5 ml/fraction) were collected by puncturing the bottom of the tube and the density of each fraction was determined using a refractometer. 100 µl samples from alternative fractions were diluted with an equal amount of TNG buffer and subjected to centrifugation at 90,000 rpm for 20 min, on a Tabletop centrifuge. The pellets were resuspended with RIPA buffer. RV proteins in the pellets, and in the sample that loaded onto the gradient was analyzed by SDS-PAGE and immunoblotting (using human anti-RV serum). RV structural protein-containing fractions were considered to be purified virus or virus-like-particle stocks.

2.2.13.3. Titration of RV

The infectivity of harvested virus or RV stock was determined using an immunochromal focus assay (Fukuda et al, 1987). Briefly, monolayers of RK cells in a 96-well plate were infected with RV in serial dilutions (10 to 10⁶) for 2 hours at 37°C. Infected cells were washed with PBS and fixed with 3% formaldehyde in PBS for 15 minutes at 72 h.p.i.. After washing twice with PBS, endogenous peroxidase was inactivated with 0.2 ml of 0.5% H₂O₂ in absolute methanol for 15 minutes at RT. The non-selective immunoglobulin binding sites of the monolayers were blocked by incubation for 1 hour at 37°C with 0.2 ml of rabbit pre-immune serum (1:200 dilution in PBS/0.5% BSA). 0.2 ml human anti-RV serum (1:200 dilution in PBS/0.5% BSA) was added and
incubated at 37°C for 60 minutes followed by three rinses with wash buffer. Cells were then incubated with peroxidase-conjugated rabbit anti-human IgG (1:200 in PBS/0.5% BSA) for one hour at 37°C and then were rinsed three times with wash buffer. Plaques were visualized after applying peroxidase substrate (0.1 ml PBS containing 0.02% cold H₂O₂ and 0.5 mg/ml 3,3’ diaminobezidine tetrahydrochloride) and incubating cells at RT till brown deposits developed. Plaques were counted from three wells per virus dilution and averaged. Titres of virus were expressed as pfu/ml.

2.2.14. Electron microscopy
For routine morphology studies, cells (RV-infected or stable transformed cells) were fixed with 2% glutaraldehyde, 3%paraformaldehyde in 100mM NaCacodylate buffer, pH 7.2, scraped from the culture dish, and pelleted in a microfuge. The cell pellets were then postfixed (one hour) in 2% OsO₄ in the same buffer, stained in block (2 hours) with 2% uranyl acetate. Dehydration was carried out with a graded series of ethanol and sample blocks were embedded in Epon plastic resin using standard methods (Barteletti et al., 1979). A series of thin (250 nm) plastic sections were collected on Formvar-coated slot grids after cut and analyzed.

2.2.15. Mice immunization
2.2.15.1. Immunization with live, purified vaccinia recombinants.
High-titer vaccinia recombinants expressing wild-type or glycosylation mutant RV E1 proteins were purified from infected cells by centrifugation in sucrose density gradients (see 2.2.6.4.). The purified recombinant viruses were titered on CV-1 cells by plaque assay (2.2.6.3) and 1x10⁵ pfu
of each recombinant (in PBS) were used to immunize individual mice (four in each group) by intraperitoneal (i.p.) injection. Mice were re-injected 4 additional times at 3 week intervals. Four mice were immunized with each vaccinia recombinant and were bled for serum 10 days after each injection. Sera from mice immunized with the same vaccinia recombinant were pooled and used for immunological assays.

2.2.15.2. Immunization with RV and virus-like particles

RV or virus-like particles were semi-purified from culture medium using centrifugation. Identical amounts of antigens (equivalent to 256 HA units) were emulsified in Freund's complete adjuvant and used to immunize mice (four in each group). Mice received three additional injections of antigens in Freund's incomplete adjuvant at three-week intervals. Mice were bled and sera were collected for analysis.

2.2.16. Enzyme linked immunoadsorbant assay (ELISA)

RV (diluted 1:600) or individual structural proteins (diluted 1:20) expressed from recombinant baculoviruses (Gillam, unpublished results) were coated onto Immulon-2 plates (Dynatech, Chantilly, VA) in carbonate buffer [15 mM Na₂CO₃, 35 mM NaHCO₃ (pH 9.5)]. Following one hour blocking in 0.5% skim milk-PBS, the plates were incubated with monoclonal antibodies (ascites fluid or tissue culture supernatant), mouse sera or human sera diluted in 0.5% skim milk-PBS. The one hour incubation was followed by the addition of alkaline phosphatase-conjugated goat anti-mouse or anti-human IgG antibodies (BRL) diluted 1:3000. The plates were developed in substrate buffer [1M diethanolamine, 5 mM MgCl₂, 2 mg/ml p-nitro-phenylphosphate (pH 9.6)]
and read at 405 nm on a Bio-Rad microplate reader (Bio-Rad, Richmond, CA).

2.2.17. Hemagglutination (HA) assay and hemagglutination inhibition (HAI) assay

HA and HAI assays were performed using a heparin/manganous chloride procedure (Liebhaber, 1970). RV antigens (25 μl) were serially diluted (two fold) with HSAG (25 mM HEPES, pH 6.5; 140 mM NaCl; 1 mM CaCl₂; 1% BSA; 0.0025% gelatin) and seeded on a polyvinyl plate. After chilling the plate at 4°C for 15 minutes, 50 μl of 0.25% one day old chick erythrocyte suspension was added to each well. Aggregation of chick erythrocytes was developed after incubation at 4°C for one hour in some wells and the HA titre of the antigen was expressed as the end-point of serial dilution at which full agglutination was observed.

For HAI assay, serum samples or ascites fluid (200 μl) were pre-treated with 200 μl of MnCl₂/heparin solution (0.5 M MnCl₂, 2500 IU/ml Porcine heparin). Following the 15 minutes of incubation, 200 μl of a 50% chick erythrocyte solution in HSAG was added and incubated on ice for one hour. An additional 600 μl of HSAG buffer was added and the serum/erythrocyte mixture was subjected to centrifugation for 10 minutes at 1,000xg and the supernatant (a dilution of 1:8) was collected. 50 μl of treated serum was serially diluted two-fold in polyvinyl plates and 25 μl of RV antigen containing 4 HA units were added to each well. Following one hour incubation at 4°C, 50 μl of 0.25% one day old chick erythrocytes in HSAG was added and the plates kept at 4°C for another hour before interpreting the results. The HAI titre was expressed as the end-point of dilution at which no aggregation of erythrocytes was observed.
2.2.18. Viral neutralization assay

Purified ascites fluid and sera from pre-immune or immunized mice were heated at 55°C for 20 minutes to inactivate complement, diluted 1:5 in M199 medium with 2% FCS, centrifuged for 10 minutes at 10,000 rpm and sterilized by filtration through a 0.22 μm pore size filter.

Serial dilutions (in triplicate) of the serum were performed in M199 medium with 2% FCS to which was added equal amounts of diluted RV (2 pfu/μl in M199, 2% FCS) with or without rabbit complement (2.5%). The virus-antibody mixture was incubated at 37°C for one hour, then 50 μl was layered onto subconfluent RK cells in 96-well microtitre plates, mixing for one hour at 37°C. The virus-antibody mixture was removed and monolayers were layered with M199 medium containing 2.5% FCS and incubated at 37°C for 72 to 96 hours. Plaques were detected using the immunoperoxidase method (2.2.13.3) and the VN titre was the reciprocal of the dilution that demonstrated at least a 50% reduction in plaque formation compared to control cells.

2.2.19. Lymphocyte proliferation assay

Lymphocytes were isolated from spleens of immunized mice. For the antigen-specific response, cells (2.5x10⁵ per well) were incubated in 96-well flat bottom plates with varying concentrations of expressed antigens in triplicate. Following 7 day incubation at 37°C with antigen, the cells were pulse labeled with [³H]-thymidine (1μCi/well) for 16 hours, harvested and washed onto glass-fibre filters with distilled water. After the filters were air dried overnight, 3 ml of Biodegradable Counting Scintillant (Amersham) scintillation fluid was added to determine the incorporation of [³H]-thymidine.
3. RESULTS and DISCUSSION

3.1. Section I. Role of N-linked glycosylation on E2 processing and transport

3.1.1. E2 cDNAs

E2 cDNAs were constructed previously in this lab (Hobman and Gillam, 1990). Oligonucleotide-directed mutagenesis was employed to introduce one or two nucleotide changes in the codons encoding asparagine or serine, resulting in a single amino acid substitution at each potential glycosylation site. The addition of N-linked oligosaccharides was prevented by changing the Asn-X-Ser consensus sequence at asparagine residues 53, 71, and 115 to Gln-X-Ser, Asn-X-Gly, and Ile-X-Ser, respectively. The mutants in which consensus sequences were altered singly are referred to as G1, G2, and G3; the double mutant is referred to as G12; and the triple mutant is referred to as G123 (Fig.5). The positions are numbered sequentially from the N-terminus of E2. The wild-type and mutant E2 cDNAs were inserted into a mammalian expression vector pCMV5 (Fig.4a) (Andersson et al., 1989) and used to transfect COS cells.

3.1.2. Determination of functional N-linked glycosylation sites in E2

N-Glycanase digestion was performed to characterize the actual number of N-linked oligosaccharide side chains on E2. N-Glycanase hydrolyses the glucosylamine linkage of all types of N-linked oligosaccharides on glycoproteins to give free oligosaccharides and polypeptides. Digestion of radio-labeled and immunoprecipitated wild-type E2 expressed in COS cells with a serially diluted N-glycanase generated four species with apparent molecular weights of 37, 35, 33, and 31K (Fig.6). It is likely that these four species corresponded to E2 with three, two, one,
Fig 5 Schematic representation of wild-type and glycosylation mutants of RV E2. The E2 protein contains three N-linked glycosylation sites at residues 53, 71 and 115 as depicted by branched structures (Y). The putative transmembrane region is located near the C-terminus of E2 (------). The first residue of mature E2 is glycine-1 and the C-terminal residue of E2 before E1 is glycine-281.
Fig. 6 Determination of the number of N-linked glycans on RV E2. [³⁵S]-methionine labeled E2 was incubated with no (lane 1); 10 mU (lane 2); 20 mU (lane 3); 50 mU (lane 4); 100 mU (lane 5) and 300 mU (lane 6) N-glycanase (Boehringer Mannheim) for 10 minutes at 37°C. E2 was separated by SDS-PAGE and subjected to fluorography. The positions of molecular weight markers are shown on the left (kDa).
and no carbohydrate side chain(s), suggesting that wild-type of E2 glycoprotein normally has three N-linked oligosaccharide chains.

3.1.3. Expression of E2 glycosylation mutants in COS cells

Analysis of the expression of E2 glycosylation mutants in COS cells was carried out according to procedures detailed in Materials and Methods. After a 30 minutes pulse-labelling period, wild-type E2 expressed as a prominent 37 kDa glycoprotein (Fig.7a, wt). The electrophoretic mobilities of the mutant proteins increased proportionally with the number of inactivated glycosylation sites (Fig.7a). Removal of any single glycosylation site at position 1, 2 or 3 resulted in the synthesis of a major 35 kDa glycoprotein, while the double mutant G23 and the triple mutant G123 directed the synthesis of proteins which migrated at 33 and 31 kDa, respectively, (Fig.7a).

To verify that the differences in electrophoretic mobility between wild-type and mutant E2 were due to the numbers of N-linked oligosaccharide side chains attached, some transfected cells were treated with tunicamycin. Tunicamycin at a low concentration efficiently inhibits N-linked glycosylation without interfering with protein synthesis in cells (Elbein, 1987). In the presence of 3 μg tunicamycin per ml, all the E2 polypeptides synthesized in cells transfected with wild-type and different glycosylation mutant cDNAs had the same molecular weight as the triple mutant, G123 (Fig.7a). Tunicamycin did not affect the apparent molecular weight of G123 from transfected cells (Fig.7a, G123; 7b, E2G123) nor did digestion with N-glycanase (Fig.7b, E2G123). These results further confirmed that all three potential N-linked glycosylation sites of E2 are normally used and that the difference in molecular weight between wild-type and mutant
Fig. 7 Expression of wild-type and glycosylation mutants of E2 in COS cells. (A). Transfected cells were labeled with [$^{35}$S]-methionine for 30 minutes in the presence (+Tm) or absence of 3 µg/ml of tunicamycin. RV specific proteins were immunoprecipitated using human anti-RV serum and separated by 11% SDS-PAGE. (B). Some immunoprecipitated E2 proteins were treated with 100 mU N-glycanase at 37°C overnight (+ glycanase) and subjected to SDS-PAGE and autoradiography. The positions of molecular weight markers are shown on the left in kDa.
E2 is due to the number of carbohydrate chain attached.

### 3.1.4. Formation of aberrant disulfide bonds in E2 glycosylation mutants

The possible formation of aberrant disulfide bonds in E2 glycosylation mutants was examined by pulse-chase analysis. Radio-labeled E2 proteins from transfected COS cells were immunoprecipitated with human anti-RV serum and separated by SDS-PAGE under reducing and nonreducing conditions (Cohen et al., 1982). Wild-type and mutant E2 proteins migrated slightly faster in the absence of β-mercaptoethanol (nonreducing) than in its presence (reducing) (Fig.8), implying the existence of intramolecular disulfide bonds in E2 that have also been observed in many other glycoproteins (Machamer and Rose, 1988a, b; Vidal, et al., 1989). The G12 protein ran as a diffuse band, and the G123 protein was not detectable on the gel under nonreducing conditions, although in the presence of β-mercaptoethanol, bands corresponding to these mutant proteins were readily detected (Fig.8). These results suggest that the formation of aberrant disulfide intramolecular bonds occurs causing the proteins to migrate as diffuse smears when disulfide bonds are not disrupted.

The possible formation of aberrant intermolecular disulfide bonds in E2 mutants was further analyzed by immunoblotting (Towbin et al., 1979). Under reducing conditions, single glycosylation mutants had a prominent 35 kDa and minor 33.5 kDa and 31 kDa glycoprotein species (Fig.9). Two species at 33 and 31.5 kDa were detected in the double mutant (Fig.9). Only the 31 kDa unglycosylated E2 protein was observed in the triple mutant (Fig.9). Under nonreducing conditions, the samples migrated slightly faster because of the presence of intramolecular disulfide bonds (Fig.9). Although the majority of E2 remained as monomer,
Fig. 8 Formation of aberrant disulfide bonding in E2 glycosylation mutants. Transfected cells were pulse-labeled with 100 μCi [35S]-methionine for 30 minutes and chased with excess methionine for 2 hours. RV-specific proteins were analyzed by immunoprecipitation using human anti-RV serum, separated on 11% SDS-PAGE with or without β-mercaptoethanol and fluorographed. The positions of molecular weight markers are shown on the left in kDa and the arrow indicates the start of the separating gel.
Fig. 9 Western blot analysis of steady-state wild-type and mutant E2 proteins in transfected cells under reducing and non-reducing conditions. Transfected COS cells were lysed (40 hour post-transfection) with RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 10 mM EDTA, 0.15 M NaCl, 0.1% SDS, 1% sodium deoxycholate) containing 10 mM iodoacetamide. Cytoplasmic extracts were electrophoresed on 11% reducing (A) and non-reducing (B) gels. The proteins were transferred to cellulose nitrate membranes. Membranes were blocked in 4% milk powder in TBS (0.15 M NaCl, 0.02 M Tris-HCl, pH 7.5) and incubated with human anti-RV serum (1:200 dilution). The proteins were visualized using alkaline phosphatase-conjugated anti-human IgG. The positions of molecular weight markers are shown on the right in kDa.
protein species corresponding to the position of E2 dimer, trimer and tetramer were readily observed (Fig.9). Deletion of any glycosylation site from E2 seemed either to abolish the binding of antibodies to E2 or reduce the amount of monomeric forms, especially in G12 and G123 (Fig.9). It is possible that these mutants proteins exist as alternatively folded structures that are not recognized by anti-RV serum and that the antigenic sites in G12 and G123 forms are detectable only after unfolding of these proteins by cleavage of intramolecular disulfide bonds. These finding suggest that the pattern of disulfide bonding for E2 glycosylation mutants is heterogeneous and the glycosylation may be important in preventing aberrant disulfide bond formation.

3.1.5. Glycan processing and intracellular stability of E2 proteins

The kinetics of processing and the turnover rate of the E2 mutant proteins were examined by pulse-chase experiments followed by densitometric analysis of processed proteins. After thirty-minute pulse-labelling, wild-type E2 was found predominantly in the 37 kDa form, and removal of high-mannose glycans by endo H digestion reduced the molecular size to 31 kDa (Fig.10). Approximately 25, 40 and 50% of wild-type of E2 was found to possess complex-type sugar after 1-, 2- and 4-hour chase periods, respectively (Fig.10). In contrast, G1, G2, and G3 mutant proteins containing complex-type glycans represented only 17, 14, and 10% of the total amount of each mutant protein after a two-hour chase (Fig.10). No endo H resistance was observed for the double mutant, G12 (Fig.10). As the acquisition of endo H resistance is believed to be indicative of transport of glycoproteins through the medial Golgi apparatus, it is evident that removal of glycosyl moieties impairs the transport of E2 mutant proteins. This effect is dependent
Fig. 10 Time course for glycan processing of wild-type and mutant E2 proteins. Cells were pulse-labeled with [35S]-methionine for 30 minutes and chased for various times as indicated. Some immunoprecipitated samples were digested with endo H for at least 8 hours (+ endo H). Endo H-resistant (R) and sensitive (S) oligosaccharide-containing proteins are indicated. The positions of molecular weight markers are shown on the left in kDa.
on both the position and the number of glycosylation sites altered.

To determine the turnover rate of wild-type and mutant E2, immunoprecipitates from transfected COS cells were fractionated on SDS-PAGE and quantitated by densitometric analysis of the autoradiographs (Fig. 11). Wild-type E2 was relatively stable in COS cells, with 70% of E2 remaining after a 4 hour chase. By contrast, the mutants exhibited a higher turnover rate. The half-lives \( t_{1/2} \) for mutant proteins in the cells were: \( G_1, G_2 \) and \( G_3 = 3 \) hours; \( G_{12} = 2 \) hours; and \( G_{123} = 30-60 \) minutes. It could be that the mutant proteins were not properly folded and transported due to an altered glycosylation pattern, and were rapidly degraded as has been reported for some other glycoproteins (Matzuk and Boime, 1988).

### 3.1.6. Intracellular localization of mutant E2 proteins.

The subcellular localization of E2 mutant proteins was examined using indirect immunofluorescence. Cells expressing wild-type E2 exhibited staining throughout the cytoplasmic reticulum as well as in the juxtanuclear region (Fig. 12a). The single, double and triple glycosylation mutant proteins displayed a predominantly reticular staining pattern as well as Golgi-like staining (Fig. 12c,e,g). To visualize the distribution of E2 protein in the ER and Golgi, fluorescent-conjugated WGA and ConA were used as markers for the compartments. WGA has been shown to label \( \text{trans} \) Golgi cisternae, associated vesicles and the cell surface (Tartakoff and Vassalli, 1983) by binding to clustered terminal N-acetylneuraminic acid residues as well as N-acetylglucosamine-containing oligosaccharide chains on glycoproteins (Virtanen et al., 1980). Co-staining of transfected COS cells with human anti-RV serum and fluorescent-conjugated WGA revealed that wild-type E2 was concentrated in the Golgi region (Fig. 12b), while the mutant E2
Fig. 11 Intracellular stability of wild-type and mutant E2 proteins. Cells were pulse-labeled with [\(^{35}\)S]-methionine for 30 minutes and chased for various times as indicated. RV-specific proteins were immunoprecipitated using human anti-RV serum. Rates of degradation of wild-type and mutant E2 proteins were quantified by scanning densitometry of the X-ray films from three to six independent experiments as shown in Fig.10. Different chase times are indicated. ---△--- wild-type, ---○--- G1, ---■--- G2, ---△--- G3, ---★--- G12, ---◆--- G123.
Fig. 12 Indirect immunofluorescence of wild-type and mutant E2 proteins in COS cells. Cells were permeabilized prior to the addition of rhodamine-conjugated WGA or ConA and anti-RV serum. After the cells were washed, a secondary antibody (fluorescein-conjugated goat anti-human IgG) was added. (a) wild-type, anti-RV; (b) wild-type, TRICT-WGA; (c) G2, anti-RV; (d) G2, TRICT-WGA; (e) G12, anti-RV; (f) G12, TRICT-WGA; (g) G123, anti-RV; (h) G123, TRICT-ConA.
proteins were distributed throughout the reticulum network and Golgi region (Fig. 12 d,f). A strong reticular staining, which co-localized with ConA, was observed in COS cells transfected with glycosylation mutants (Fig. 12h). In addition, unlike wild-type E2, which has been shown to exhibit limited amount of cell surface expression (Hobman and Gillam, 1989), the glycosylation mutants had no detectable cell surface signal (data not shown). Elimination of any of the glycosylation sites in E2 seemed to impair the intracellular transport and to block the cell surface expression of E2.

3.1.7. Secretion of an anchor-free form of wild-type and mutant E2 proteins.

To analyze the transport behavior of E2 mutants in the secretory pathway, a panel of truncated E2 glycosylation mutants were constructed, each of which had 68 amino acids deleted from the hydrophobic C-terminus (Hobman et al., 1994). The truncated form of wild-type E2 was secreted into the culture medium as a 36-kDa endo H-resistant glycoprotein (Hobman et al., 1994). The truncated form of E2 single glycosylation mutants (G1, G2 and G3) but not double (G12) and triple (G123) mutants were also secreted into the culture medium, although not as efficiently as the anchorless wild-type E2 (Fig. 13a). In addition, the efficiency of secretion appeared to depend on the position of the deleted glycosylation site. Deletion of the glycosylation site proximal to the C-terminus (G3) had a more profound inhibitory effect on the secretion than of the central site (G2) and of that proximal to the N-terminus (G1) (Fig. 13a).

The intracellular forms of anchorless wild-type E2 and E2 glycosylation mutants were sensitive to endo H digestion (Fig. 13b), whereas the secreted E2 was endo H-resistant (Fig. 13a). Expression of the truncated triple mutant was not detected intracellularly (Fig. 13b). The 31-kDa
Fig. 13 Intracellular processing and secretion of a soluble form of wild-type and mutant E2 proteins. Cells were labeled with \([^{35}\text{S}]\)-methionine for 30 minutes and chased for 4 hours. (a). Immunoprecipitated samples from culture media of cells transfected with anchorless wild-type and mutant E2 cDNA constructs. (b). Intracellular forms of each anchorless E2 protein of wild-type and glycosylation mutant. Equal volumes of each sample were incubated at 37°C for at least 8 hours with or without endo H and separated on 11% SDS-PAGE. The positions of the molecular weight markers are shown on the left in kDa.
endo H-sensitive protein species found in the culture medium of the G2 transfected cells (Fig.13a) is probably due to lysis of a portion of cells during the chase period and the intracellular G2 mutant protein was released into the medium. Taken together, it was evident that the single glycosylation mutants G1, G2 and G3 were transported out of the ER through the Golgi to the cell surface and then exited the cell into the culture medium, although not as efficiently as the otherwise unaltered anchorless E2 protein.

3.1.8. Summary and Discussion

The role of N-linked glycosylation in processing and intracellular transport of RV E2 glycoprotein has been studied by expressing glycosylation mutants of E2 in COS cells. In RV M33 strain, all three sites were used for the addition of N-linked oligosaccharides. Removal of any of the glycosylation sites resulted in slower glycan processing, lower stability and aberrant disulfide bonding of the mutant proteins, with the severity of the defect depending on the number of deleted carbohydrate sites. The mutant proteins were translocated into the ER and transported to GC but were not detected on the cell surface. However, the secretion of the anchor-free form of E2 into the medium was not completely blocked by the removal of any one of its glycosylation sites. This effect was dependent on the position of the deleted glycosylation site.

Protein movement from the ER to the medial Golgi apparatus has been identified as the rate-limiting step in the exocytic pathway (Rose and Bergmann, 1983), as measured by the acquisition of a variety of organelle-specific post-translational modifications. Regarding the intracellular transport rate, several viral glycoproteins that have been extensively investigated fall into two categories. The first group includes the VSV G protein (Rose and Doms, 1988) and
influenza virus HA (Gallagher et al., 1988) which move quickly along the exocytic pathway. After a 15 minutes chase period, 50% of the oligosaccharides on VSV G and 25% on influenza virus HA acquire endo H resistance (Rose and Doms, 1988; Gallagher et al., 1988). The second group includes the HIV envelope protein (Earl et al., 1991) and simian virus 5 hemagglutinin-neuraminidase (SV5 HN) (Ng et al., 1989), for which acquisition of endo H resistance was observed within 80 minutes and 60 minutes post-labelling, respectively. We found that the carbohydrates on wild-type RV E2 were converted to complex-type sugar moieties by 1 hour post-labelling. However, the conversion was not complete even after a 8 hours chase period (data not shown), reflecting a slow movement of RV E2 from the ER to the Golgi apparatus.

The data from this study showed that E2 contains three potential oligosaccharide addition sites and all three potential N-linked glycosylation sites were utilized (Fig.6 and 7). Inactivation of these functional sites impaired the processing as well as the intracellular stability of E2 proteins (Fig.10 and 11), the severity of the defect depending on both the number and the position of the glycosylation site deleted. Deletion of one N-linked glycosylation site on RV E2 considerably reduced the rate of transport, as determined by the fraction of proteins that acquired endo H-resistant carbohydrates (Fig.10). The glycosylation site proximal to the N-terminus (G1) seems to be less important than the site proximal to the C-terminus (G3), as judged by the fraction of molecules containing endo H-resistant carbohydrates for the membrane-bound form and by the secretion ratio of their anchorless counterparts (Fig.10 and 13). That the oligosaccharide at each glycosylation site may play a different functional role has been noted previously with other glycoproteins (Matzuk and Boime, 1988; Ng et al., 1990). In addition, studies on other glycoproteins using the same approach have shown that glycosylation on all the
predicted sites is not a prerequisite for folding, assembly and transport of the protein (Guan et al., 1988). It has been suggested that the contribution of each carbohydrate chain varies depending on its location in a different conformational circumstance of a particular protein. RV E2 is rich in cysteine and undergoes intramolecular disulfide bonding (Fig.9). Inspection of the amino acid sequence of RV E2 reveals that the G2 and G3 glycosylation sites are flanked by two cysteine residues (Clarke et al., 1987). It is possible that the oligosaccharides attached to the G2 and G3 sites are important in preventing improper intramolecular disulfide bond formation, whereas glycosylation at the G1 site has less effect on proper folding and transport. The diffuse or smeared appearance of non-reduced mutant G12 and G123 proteins on an immunoblot (Fig.9) probably reflects the formation of aberrant intermolecular disulfide bonds. Thus, it appears that oligosaccharide addition is required for proper intramolecular disulfide bonding to promote correct folding, which in turn is essential for efficient transport (Vidal et al., 1989). Removal of a glycosylation site leads to formation of improper intramolecular disulfide bonds and protein misfolding. Dramatic alteration in protein conformation and possibly aggregation could be the consequence when glycosylation sites are inactivated. This may account for diminished antibody binding by G12 and G123 proteins under non-reducing conditions (Fig.9B).

Proteins that are transported slowly in cells display heterogeneity in endo H resistance. For example, this has been observed for SV5 HN (Ng et al., 1989), influenza virus neuraminidase (Kunda et al., 1991) and HIV gp120 (Earl et al., 1991). Pulse-chase experiments have demonstrated that endo H-sensitive, partially endo H-resistant and endo H-resistant E2 forms represent the ER-, Golgi- and cell surface- isoforms of RV E2. Immunofluorescence of transfected COS cells expressing E2 wild-type and mutant genes showed that the majority of the
glycosylation mutant proteins were localized in the ER (Fig.12). A small fraction were found in
the Golgi region (Fig.12). Transport of the E2 single glycosylation mutants into the Golgi
compartment was evidenced by the presence of partially endo H-resistant bands after the chase
period (Fig.10), as well as by the secretion of the C-terminal truncated form of E2 single
glycosylation mutants (Fig.16a). Thus the transport of E2 to the Golgi apparatus appeared to be
significantly affected but not completely blocked by the absence of any one of the N-linked
oligosaccharides.

The anchorless E2 single glycosylation mutants were secreted into the culture medium,
although less efficiently than wild-type anchorless E2. The oligosaccharides on the secreted forms
of wild-type and mutant E2 were completely endo H-resistant as is E2 from RV virion
(Lundstrom et al., 1991), suggesting that carbohydrates attached to these proteins are modified
by Golgi enzymes. This finding indicates that the soluble forms of E2 single glycosylation
mutants are transported through the normal exocytic route. Inability to detect cell surface
expression of E2 single glycosylation mutants could be due either to the low sensitivity of
indirect immunofluorescence in our experiments, or to the fact that mutant E2 proteins were
quickly and extensively internalized from the plasma membrane, as has been observed for other
glycoproteins (Ng et al., 1989).
3.2. Section II. Effect of Brefeldin A (BFA) and monensin on protein processing and virus assembly

3.2.1. Processing of N-linked oligosaccharides on E2 in BFA- and monensin-treated cells

The expression of RV E2 in pCMV5-E2 transfected COS cells was used to determine the appropriate concentrations of BFA and monensin for the study. Interestingly, BFA seemed to enhance the level of E2 protein expression (Fig.14A), the mechanism of which is not understood. However, no 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.14A) and thus 6 μg/ml of BFA was chosen for use in the subsequent analysis. In contrast, monensin at higher concentrations (25 μM) inhibited E2 synthesis (Fig.14C), and hence 5 μM monensin was used in the subsequent experiments.

Endo H, an enzyme that removes N-linked high-mannose oligosaccharide, was used to monitor the processing of E2 glycoproteins in pulse-chase experiments. In control (untreated) cells after 30 minutes labelling, E2 accumulated as a 37 kDa protein (Fig.14A). Digestion with endo H reduced the molecular size of the protein species to 31 kDa (Fig.14A). By contrast, in BFA-treated cells, only the 42 kDa species was observed by the end of 30 minutes labelling (Fig.14A) and digestion with endo H decreased its molecular weight from 42 kDa to 36 kDa (Fig.14A). By 4 hour post-labelling, in control cells, about 50% of synthesized E2 was partially endo H-resistant (Fig.14C). Whereas BFA induced a rapid and complete conversion of glycans on E2 to endo H-resistant forms by 4 hour post-labelling (Fig.14C). In monensin-treated cells, pulse-labeled E2 protein had a similar electrophoretic mobility to that of the control cells (Fig.14B). However, after 3 hours of chase, monensin treatment gave rise to a novel E2 protein.
Fig. 14 Effect of BFA and monensin on processing of E2. Transfected cells were pulse-labeled with 100 μCi [35S]-methionine for 30 minutes and incubated with excess methionine for indicated time in chase experiments (C, D). BFA (in μg) (A) or monensin (in μM) (B) were added to the medium at the concentrations shown on top of A and B. In chase experiments, 6 μg/ml of BFA (C) or 5 μM of monensin (D) were present. Cells were lysed with RIPA buffer after labelling (A and B) or after chase for the indicated period of time (C, D). E2 proteins were immunoprecipitated with human anti-RV serum, subjected to SDS-PAGE and fluorography. Half of each immunoprecipitated sample was digested with endo H (+) for 8 hours at 37°C before separating on SDS-PAGE. Molecular weight markers (in 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 protein species after endo H digestion is indicated by a star (*).
band with partial endo H-resistant sugar moieties and an apparent molecular weight of 34 kDa (Fig. 14D, star) instead of 36 kDa seen in control cells (Fig. 14D, ctrl).

### 3.2.2. Processing of O-linked glycans on E2

Since it has been shown that E2 contains O-linked oligosaccharides (Lundström et al., 1991; Sanchez and Frey, 1991), I then analyzed the extent of O-glycosylation of E2 in BFA- and monensin- treated cells and in cells also treated with tunicamycin. In control cells (untreated with BFA nor monensin) after a 4 hour chase period, digestion with endo F/PNGase F reduced the molecular weights of two E2 species from 42 kDa and 37 kDa to 36 kDa and 31 kDa, respectively (Fig. 15A), similar to that observed after endo H digestion (Fig. 14C). After incubation with neuraminidase, the 37 kDa remained unchanged whereas the 42 kDa species was reduced to 40 kDa (Fig. 15A). This 40 kDa species was diminished after further digestion with O-glycosidase (Fig. 15A). Deglycosylation of E2 with the combination of endo F/PNGase F, neuraminidase and O-glycosidase resulted in a major unglycosylated 31 kDa species and a 34 kDa minor band probably due to incomplete removal of O-linked oligosaccharide (Fig. 15A). 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. 15A). The cause for the 1 kDa difference in molecular weight between deglycosylated E2 (by glycosidase digestion) and unglycosylated E2 (by tunicamycin treatment) is not known.

In BFA-treated cells, the 42 kDa E2 species was reduced to 36 kDa by endo F/PNGase F digestion, to 39 kDa by neuraminidase digestion, or to 37 kDa by further incubation with O-glycosidase (Fig. 15B). Digestion with a combination of endo F/PNGase F, neuraminidase and O-
Fig. 15. Glycosidase digestion of E2 from BFA- and monensin-treated cells. Transfected cells were labeled with [35S]-methionine for 30 minutes and incubated with medium containing excess methionine for 4 hours before lysis with RIPA buffer. E2 proteins were immunoprecipitated with human anti-RV serum, digested with glycosidase for at least 8 hours 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. Samples digested with endo F/PNGase F (N-gly), neuraminidase (Neu) and O-glycosidase (O-gly) are indicated. Molecular weight markers (in kDa) are included for reference. A, untreated cells. B, cells treated with BFA. C, cells treated with monensin. Tunicamycin was present in the medium of some cells as indicated.
glycosidase resulted in a single protein species of 31 kDa (Fig.15B). In cells treated with both tunicamycin and BFA, two protein species with apparent molecular weights of 31 kDa and 35 kDa were observed (Fig.15B). 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 weight to 33 kDa while further incubation with O-glycosidase gave rise to a single protein species of 30 kDa (Fig.15B).

E2 from monensin-treated cells migrated as a broad band with molecular weights ranging from 37K to 42K (Fig.15C). The presence of 36 kDa E2 species in endo F/PNGase F treated samples indicated that O-linked glycosylation occurred (Fig.15C), in contrast to the situation seen in some other viral glycoproteins (Ogura et al., 1991; Collin and Mottet, 1992). A distinctive protein species with molecular weight 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.15C). This could be an intermediate during processing of O-linked glycans that accumulated in monensin-treated cells or alternatively is due to incomplete removal of O-linked sugars from E2 in an altered conformation induced by monensin-treatment.

In cells treated with monensin and tunicamycin, no O-linked glycosylation occurred (Fig.15C).

Taken together, these data suggested that E2 was first synthesized in the ER as a 37 kDa protein species containing only N-linked high-mannose type of glycans, and that as it reached the Golgi cisternae, O-linked glycosylation took place which increased the molecular weight of E2 to 42 kDa. BFA treatment caused a redistribution of Golgi enzymes back 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. Whereas in monensin-treated cells, the O-
glycosylation of E2 was not abolished, but was processed aberrantly.

3.2.3. Processing and secretion of an anchor-free form of E2.

To study the transport of E2 along the secretory pathway in BFA and monensin treated cells, a cDNA construct encoding an anchor-free form of E2 (Hobman et al., 1994) was transfected into COS cells, and the expressed E2 protein was analyzed. 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.16), 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.16). In contrast, although the internal form of soluble E2 in BFA treated cells was partially or completely resistant to endo H digestion (Fig.16), no secreted E2 was detected (Fig.16). Monensin completely inhibited the secretion of E2 from transfected cells, whereas intracellular E2 exhibited no obvious difference from that of control cells (Fig.16). Taken together, it is evident that BFA and monensin cause altered oligosaccharide processing during E2 transport and completely block the movement of E2 to the cell surface.

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

The expression of the polyprotein precursor and the proteolytic processing of the precursor were analyzed by pulse-chase experiments using pCMV5-24S (Hobman et al., 1990) transfected COS cells. At the end of a 30 minutes labelling, the majority of RV structural proteins were present
Fig. 16 Effect of BFA and monensin on processing and secretion of an anchor-free form of E2. COS cells were transfected with plasmid containing cDNA encoding a secreted form of E2 and labeled with 100 μCi [35S]-methionine for 30 minutes at 40 hour post-transfection. After incubation with excess methionine for 4 hours, 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 fluorography. Half of each sample was digested with endo H (+). Molecular weight markers (in kDa) are shown on the left. Ctrl, E2 from untreated cells; BFA, E2 from BFA-treated cells; mon, E2 from monensin-treated cells.
Fig.17 Effect of BFA and monensin on the proteolytic cleavage of the polyprotein precursor for RV structural proteins. Cells transfected with pCMV5-24S were pulse labeled with [35S]-methionine and chased for 3 hours. RV structural proteins were recovered from cell lysates by immunoprecipitation with human anti-RV serum and were subjected to SDS-PAGE and fluorography. The bands corresponding to RV structural proteins without endo H treatment (on the left) and with endo H treatment (on the right) are indicated. E2r, E2 proteins that contain endo H-resistant glycans; E2s, E2 that contain endo H-sensitive sugar moieties. Endo H digestion is indicated as +. Ctrl, untreated cells; BFA, BFA treated-cells; mon, monensin-treated cells.
as individual polypeptides, although some E1/E2 uncleaved precursor protein species with higher molecular weights were also observed (Fig.17, pulse). These minor protein species were not seen after a 3 hour chase period (Fig.17, chase). There was no significant difference in cleavage efficiency 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 carried out by cellular signal peptidases (McDonald et al., 1991; Qiu et al., 1994), which suggests that it is an ER-specific event and was not interrupted by the influx of resident Golgi proteins upon BFA treatment or an impaired Golgi upon monensin treatment. RV structural proteins appears to assemble into virus-like particles in the absence of genomic RNA and are released from the cells (see 3.4.), which probably is the reason that there was a slight decrease in the amount of RV structural proteins in control cells during chase.

3.2.5. Subcellular distribution of RV structural proteins in BFA and 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 and co-localized with a Golgi marker (Fig.18a,b), while E1 was localized in the peri- and juxtanuclear region corresponding to the ER and Golgi structure (Fig.18c,d). The capsid protein was distributed throughout the cytoplasm (Fig.18e). A limited amount of E2 and E1 was detected at the cell surface (Fig.18f). In BFA treated cells, E2 and E1 displayed a predominant perinuclear staining pattern which was co-localized with a Golgi marker (Fig.18, g-k). Combined with the
Fig. 18 Indirect immunofluorescence of RV structural proteins in cells transfected with pCMV5-24S and treated with BFA or monensin. Cells were permeabilized prior to the addition of rhodamine-conjugated anti-Golgi protein serum, mouse monoclonal antibodies or human anti-RV serum. Some samples (F, L, and R) were not permeabilized for detection of cell surface expression. After cells were washed, a secondary antibody (fluorescein-conjugated goat anti-mouse or anti-human IgG) was added. a-f, control cells: a) anti-E2; b) same cell, anti-Golgi; c) anti-E1; d) same cell, anti-Golgi; e) anti-C; f) anti-RV, cell surface. g-l, transfected cells treated with BFA: g) anti-E2; h) same cell, anti-Golgi; i) anti-E1; j) same cell, anti-Golgi; k) anti-C; l) anti-RV, cell surface. m-r, transfected cells treated with monensin: m) anti-E2; n) same cell, anti-Golgi; o) anti-E1; p) same cell, anti-Golgi; q) anti-C; r) anti-RV, cell surface.
results from pulse-chase analysis, it appeared that in transfected COS cells, BFA treatment caused the Golgi 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.18n,p), along with a diffuse E2 and E1 distribution in the cytoplasm (Fig.18m,o). Capsid protein was distributed in the cytoplasm in BFA and monensin treated cells (Fig.18k,q). No cell surface fluorescence was detectable in BFA and monensin treated cells (Fig.18l,r).

3.2.6. Effect of BFA and monensin on RV assembly and release

Release of radio-labeled RV particles was monitored in RV-infected Vero cells treated with BFA and monensin (see Material and Methods). In control (untreated) cells, the amount of viral structural proteins (C, E2, E1) increased with time during the chase period (Fig.19), indicating that virus particles were accumulating and that viruses were steadily assembled and released from the cells. In BFA and monensin treated cells, no viral structural proteins were detectable until 36 hours post-labelling (Fig.19). Thus, BFA and monensin blocked RV release from the cells during the early stage of the chase. After a 36 hour chase period, the viral proteins detected may be due to lysis of BFA and monensin treated cells (Fig.19).

To further address this question, a one-step growth experiment was performed with RV infected Vero cells treated with BFA and monensin. BFA and monensin were added to the medium 8 h.p.i. and maintained for 60 hours. Cell-associated virus and virus in the medium were titred (Fig.20). In control cells, intracellular and extracellular viruses reached the highest titre at
Fig. 19 Release of virus particles from infected cells. Vero cells were infected with RV at a MOI of 10 and labeled with \[^{35}S\]-methionine for 1 hour at 24 h.p.i. Cells were further incubated in normal medium for the indicated times. Virus particles were recovered from the medium by polyethyleneglycol precipitation, resuspended in RIPA buffer and subjected to SDS-PAGE. RV structural proteins are indicated by arrowheads. Ctrl, untreated cells; BFA BFA-treated cells; Mon, monensin-treated cells.
Fig. 20 Titration of cell-associated and released virus. Vero cells were infected with RV at a MOI of 10 and incubated for the indicated time. Medium samples were collected and cells were subjected to freezing and thawing three times to release intracellular virus. Virus infectivity was titrated on RK cells and is expressed as PFU/ml (detailed in Materials and Methods). ■, intracellular virus titre. □, extracellular virus titre.
48 h.p.i, about $5 \times 10^7$ pfu/ml, with a slightly higher extracellular virus than intracellular titre (Fig.20). Titres of cell-associated viruses were much lower in BFA and monensin treated cells, about $2 \times 10^5$ pfu/ml (Fig.20), indicating that BFA and monensin dramatically reduced virus assembly in infected cells. Titres of extracellular virus from BFA- and monensin-treated cells represented only 0.1% of the total virus (Fig.20). Thus, BFA and monensin effectively inhibited virus release from infected cells. Infected cells became unhealthy with prolonged monensin treatment as an aberrant cell morphology was observed. This could explain the increased extracellular virus titre after incubation for 60 hours (Fig.20).

3.2.7. Assembly of virus particles in control or BFA and monensin treated cells

RV particles in infected cells were visualized using conventional electron microscopy. Cells were fixed at 48 h.p.i., dehydrated and embedded. Thin sections were examined after staining. In RV-infected Vero cells, virions were predominantly located in vacuoles in the proximity of the Golgi cisternae (Fig.21b) and in large transport vesicles (Fig.21a). Unenveloped nucleocapsids were rarely observed in the cytoplasm and no virus budding at the plasma membrane was observed after examining all the sections. In BFA-treated cells, the GC was disassembled and resulted in a dilated ER structure (Fig.21c). The number of enveloped virus particles was dramatically decreased and they were predominantly located in the cytoplasm, not associated with any membrane structure (Fig.21c). In monensin-treated cells, enlarged vesicles were observed (Fig.21d). No virus particles were found except that electron-dense particles, comparable in size to RV nucleocapsid, was observed in enlarged vesicles (Fig.21d).
Fig. 21 Electron microscopic analysis of virus assembly. Monolayers of Vero cells were infected with RV at a MOI of 10 and some were treated with BFA or monensin. At 48 h.p.i., cells were fixed and prepared for electron microscopic analysis. A) RV-infected cells showing virus accumulated in large vacuole, x75,000. B) RV infected-cells showing virus maturation in the proximity to the Golgi stack, x25,000. C) RV infected-cells treated with BFA. Virus particle was found in the cytoplasm, near a dilated ER structure. x25,000. D) RV infected cells treated with monensin. No virions were observed. Electron dense particles were found in large vesicles. x25,000. er, endoplasmic reticulum; Gc, Golgi complex; nu, nucleus.
3.2.8. Summary and Discussion

The effect of BFA and monensin on the transport and processing of RV structural proteins as well as virus assembly and release was examined. BFA and monensin effectively blocked the cell surface expression of RV E2 and E1 membrane glycoproteins and the secretion of an anchor-free form of E2. A dramatic change in the intracellular distribution of RV structural proteins was also observed, although the proteolytic processing of RV structural protein precursor was not affected. In the presence of BFA and monensin, virus release from infected Vero cells was only 0.1% of the intracellular virus. Virus particles were observed predominantly in large vesicles or Golgi stacks in RV-infected Vero cells but were found in the cytoplasm in BFA treated cells.

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 the appropriate cellular compartment have been found to significantly reduce the formation of infectious virus particles (Haggerty et al., 1991). In the Togavirus family, RV differs from the other subgroup (alphavirus) in that it has a relatively long latency period, slow replication and low virus yield in cultured cells (reviewed by Porterfield et al., 1978). This is despite the fact that these two subgroups share similar virion structure as well as strategies for viral gene expression. The transport rate of RV glycoproteins in transfected cells is fairly low compared to that of the envelope glycoproteins from SFV and SIV, the two prototypes of alphaviruses. In cells transfected with RV E2 cDNA, about 50% of E2 contains endo H-resistant sugar moieties after a 4 hour chase period (Fig.14), indicating that E2 moves slowly from the ER to the Golgi stack.
Immunofluorescence studies show that the majority of RV glycoproteins E1 and E2 are concentrated in the ER-Golgi region. The abundance of RV glycoproteins in the GC may be crucial to the virus' capacity to bud from intracellular membranes (Bardeletti et al., 1979).

In RV virion, E2 glycoprotein exists as multiple protein species with molecular weights ranging from 42K to 47K. It has been suggested that this is due to heterogeneous glycosylation. In transfected COS cells, a 37 kDa E2 protein with endo H-sensitive sugars and a 42 kDa E2 protein with partial endo H-resistant glycans were observed. The amount of the 42 kDa protein species increased with time in a pulse-chase experiment. Thus, it has been proposed that the 42 kDa protein represents the Golgi-form of E2 while the 37 kDa protein is the form present in the ER (Hobman and Gillam, 1989) and that the partial resistance of E2 to endo H digestion reflects the slow transport rate of E2. However, employing both O-glycosidase and endo F/PNGase F to remove O- or N-linked oligosaccharides on E2 separately, I 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 (Fig.15). The endo H-resistant sugar moieties present on the 42 kDa protein, in fact, are the O-linked glycans. These data suggest that E2 is first synthesized as a 37 kDa protein with only N-linked high-mannose sugars in the ER, and is 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 with both N- and O-linked sugars was observed (Fig.15, 16). This probably results from the addition of O-linked glycans to the 37 kDa protein normally residing in the ER by the Golgi O-glycosylation enzymes 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 forms was slower, as judged by the fact that complete endo-H resistant E2 was observed after a 4 hour chase. Although monensin appeared to abolish O-glycosylation in many cases (Colins and Mottet, 1992), I 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 and monensin. In BFA treated cells, the Golgi complex was disassembled as the Golgi marker was localized in the perinuclear space (Fig.18h, 18j). A dilated Golgi morphology was found in monensin treated cells (Fig.18n, 18p). There was a consistent co-localization of RV membrane glycoproteins with the Golgi markers, suggesting that RV envelope glycoproteins interact strongly with Golgi macromolecules. Recently, it has been shown that unassembled subunits of RV E1 glycoprotein are arrested in a post-ER, pre-Golgi compartment (Hobman et al., 1992); however, co-expression of E1 and E2 could lead to the release of E1 from this retention and allow targeting to the GC (Hobman et al., 1993). The implication of these observation is that folding and multimerization of RV glycoproteins is a slow, albeit important event necessary for transport of E1 and E2 out of the ER. On the other hand, although the mechanism underlying retention in the Golgi is not well understood, hydrophobic domains which may specify residence in the Golgi stack (Swift and Machamer, 1992), are present in both E1 and E2 primary structure.

In RV-infected Vero cells, virions were steadily assembled and released into the medium, as judged by titres of virus in the medium and in association with cells (Fig.20). Slightly more extracellular virus than intracellular was found (Fig.20), which is consistent with earlier reports (Payment et al., 1975; Bardeletti, et al., 1979). In BFA and monensin treated cells, very few
infectious virus particles in the medium were detected (Fig.20), indicating that BFA and monensin effectively blocked the production of extracellular virus. The absence of radio-labeled virions in the medium of BFA and monensin treated cells (Fig.19) ruled out the possibility that the decreased virus titre was due to the loss of virus infectivity. Rather, it was due to a decrease in the number of virus particles during treatment. A dramatic decrease in intracellular virus titre was also observed (Fig.20). This could be due to the inhibition of virus assembly at the internal membrane or aberrant virus assembly, which is yet to be investigated. Recently it has been reported that BFA affected viral RNA synthesis in poliovirus infected cells (Maynell et al., 1992) due to interruption of membrane-associated poliovirus replication complex. The nature and site of RV RNA synthesis is not well known. However, we found that the level of protein expression in cDNA-transfected and RV-infected cells was not affected by the concentration of BFA and monensin used (data not shown).

In Vero cells, envelopment of RV nucleocapsid to form virions has been reported to take place both at the internal membrane or at the cell surface (Payment et al., 1975). In this study, morphological analysis was performed in RV-infected cells in the presence and absence of BFA and monensin in order to examine the correlation between viral glycoprotein targeting and the virus budding process. At 48 h.p.i., virus particles were found to be clustered in the vacuoles near the Golgi stack, with others in the transport vesicles (Fig.21). The abundance of virus particles in the post-Golgi structures suggested that the post-Golgi membrane network may be the primary source of membrane in RV assembly. Few virus particles, not associated with any membrane structure, were found in the juxtanuclear region of cytoplasm in BFA treated cells (Fig.21). No virus particles could be detected in monensin treated cells after examining all the sections
(Fig.21). The results from morphological analyses were consistent with a significant decrease in virus titre in BFA and monensin treated cells. The reduction of intracellular virus assembly in BFA or monensin treated cells could be due to the blockage of glycoprotein transport to the site of envelopment or the disruption of vesicular structure which may be required for efficient virus assembly. Taken together, the data presented in this study suggested a correlation between intracellular localization of RV structural proteins and the site of RV assembly.

How cell membranes become incorporated into the envelope of the virion is as yet poorly understood. The subcellular location for acquiring membranes differ with viruses, reflecting the possibility that structural or component features of particular organelles are required to facilitate such envelopment. Alternatively, post-translational modification(s) on viral envelope proteins during transport may be essential for effective interaction with the nucleocapsids. Therefore, defining the site of virus assembly can shed some light on further studies on the mechanism of virus assembly.
3.3. Section III. Influence of N-linked glycosylation on the antigenicity and immunogenicity of E1 glycoprotein

3.3.1. Construction of recombinant vaccinia viruses expressing RV E1 glycosylation mutants

The cDNA fragments encoding RV E1 glycosylation mutants (Hobman et al., 1991) were subcloned into the Sma I site of vaccinia virus recombination vector pGS20 (Fig.4B), which contains the conventional p7.5 early/late promoter (Mackett et al., 1984). Transfection (see Materials and Methods 2.2.6.) and marker rescue by cell-mediated homologous recombination were performed as described by Mackett et al., (1985). The vaccinia recombinants containing wild-type and glycosylation mutant cDNAs were named according to the mutations as VR-E1 (wild-type); VR-E1G1, VR-E1G2, VR-E1G3 (single mutations); VR-E1G23 (double mutation) and VR-E1G123 (triple mutation) (Fig.22a). The construction of a vaccinia recombinant expressing RV E2 has been described (Chaye et al., 1992b).

3.3.2. Expression and antigenicity of E1 glycosylation mutants.

Cells infected with the wild-type E1 vaccinia recombinant expressed a 57 kDa protein (Fig.22b, E1), while the vaccinia recombinants containing E1 glycosylation mutant cDNAs expressed proteins with apparent molecular masses of 55 kDa (Fig.22b, G1, G2, G3), 53 kDa (Fig.22b, G23) and 51 kDa (Fig.22b, G123), similar to those observed in COS cells (Hobman et al., 1991). The differences in electrophoretic mobility between the wild-type and glycosylation mutants were due to differences in the numbers of oligosaccharides attached to the wild-type and mutant proteins. Corresponding polypeptides were absent from uninfected cells (Fig.22b, M) and cells
Fig. 22a. Diagrammatic representation of RV E1 glycosylation mutant cDNAs used in the construction of vaccinia recombinant viruses. The respective portions of the E2 and E1 genes are indicated above the constructs. The translation initiation site is contained in the region proceeding the N-terminus of capsid near the left end of the constructs. Three N-linked glycosylation sites at residues 76, 177, and 209 are indicated by Y and the mutagenized glycosylation sites are marked G1, G2, and G3. The EcoRI (E) and HindIII (H) sites flanking the 5' and 3' portion of the cDNAs, respectively, are indicated. The thick horizontal lines represent coding regions and the thin lines indicate noncoding regions. The thick vertical lines demarcate the regions of C, E2 and E1 coding regions (Hobman et al., 1991).
Fig. 22b. Expression of E1 glycosylation mutants by vaccinia recombinants. CV-1 cells infected with wild-type vaccinia virus (WT), RV E1 vaccinia recombinants (E1, G1, G2, G3, G23 and G123) or mock infected (MI) were labeled with $[^{35}\text{S}]$-methionine for 30 minutes at 10 h.p.i. Cells were lysed with RIPA buffer and RV E1-specific proteins were immunoprecipitated with RV E1 mAbs and subjected to SDS-PAGE. Molecular weight marks in kDa are shown on the right for reference.
infected with wild-type vaccinia virus (Fig.22b, WT).

To characterize the immunoreactivity of the E1 mutant proteins, a panel of RV E1-specific mAbs was used for western blot analysis under reducing and non-reducing conditions. Six RV E1-specific mAbs (21B9H, 3D5D, 3D9F, 14D1F, 16B2C and H4C52) were used in this analysis. The mAbs were characterized as having VN (21B9H) or HAI (3D9F, 3D5D, 16B2C, H4C52) activities (Chaye et al., 1992a). Similar results were obtained from all six mAbs used and Fig.23 shows two typical blots using mAbs 21B9H and 3D9F. Under reducing conditions (Fig.23, + β-Me), all five E1 glycosylation mutants reacted with each of the six mAbs, suggesting that these mAbs recognize linear epitopes on the E1 primary structure. The capacity of these mAbs to bind to non-glycosylated E1 (Fig.23, + β-Me, G123) indicated that these epitopes were not carbohydrate-dependent. The weak signal observed in immunoblots under reducing conditions is due solely to the decrease in antigenicity of E1 and not to the quantitative difference in the antigen used. Under non-reducing conditions, the majority of the wild-type and the single glycosylation mutants ran as monomers with a small fraction as dimers (Fig.23, - β-Me, wt, G1, G2 and G3), while the double mutant G23 gave a smeared appearance on the gel (Fig.23, - β-Me, G23) and the triple mutant G123 was not detectable (Fig.23, - β-Me, G123). One possible explanation for this observation is that mutant proteins G23 and G123 form aberrant intermolecular disulfide bonds that cause them to migrate as diffuse smears on SDS-PAGE. G123 was not present on the top of the separating gel. Taken together, the data presented here suggest that denaturation of mutant proteins G23 and G123 in the absence of β-mercaptoethanol led to decreased or abolished antibody binding activity of these proteins. Decreased antibody binding was also observed in native G23 and G123 proteins in immunoprecipitation (Fig.22b).
Table 1a. Summary of properties of monoclonal antibodies directed to E1

<table>
<thead>
<tr>
<th>mAbs</th>
<th>VN or HAI</th>
<th>Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>21B9H</td>
<td>VN</td>
<td>E1_{214} to E1_{233}</td>
</tr>
<tr>
<td>H4C52</td>
<td>HAI</td>
<td>ND</td>
</tr>
<tr>
<td>3D5D</td>
<td>HAI</td>
<td>ND</td>
</tr>
<tr>
<td>14D1F</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3D9F</td>
<td>HAI</td>
<td>E1_{214} to E1_{240}</td>
</tr>
<tr>
<td>16B2C</td>
<td>HAI</td>
<td>ND</td>
</tr>
</tbody>
</table>
Fig. 23 Immunoblot analysis of E1 glycosylation mutants expressed by vaccinia recombinants. Cells infected with vaccinia recombinants expressing wild-type E1 (WT) or E1 glycosylation mutants (G1, G2, G3, G23, G123) were treated with 10 mM iodoacetamide and lysed with RIPA buffer. Proteins were separated on SDS-PAGE under reducing (+ β-Me) and non-reducing (- β-Me) conditions, and transferred to nitrocellulose membrane. RV antigens were probed with a panel of RV E1-specific mAbs. Parental vaccinia virus infected cell lysate was used as the control (ctrl). Molecular weight markers (in kDa) are included for reference.
Therefore glycosylation may be required for proper folding of E1 to allow efficient recognition of immunological epitopes on E1.

3.3.3. Immunogenic properties of the expressed E1 glycosylation mutants.

Although glycosylation did not significantly affect antibody binding, it might affect the elicitation of HAI and VN antibody responses. To address this question, vaccinia recombinant viruses were purified and used to immunize mice. After four injections, sera were collected, pooled and tested for their reactivities to RV E1 by immunoblot analysis. All the E1 glycosylation mutants except G123 were found to elicit antibodies recognizing authentic E1 from RV virions when RV proteins were separated under non-reducing conditions (Fig. 24, - β-Me). However, only the antiserum from wild-type E1 immunized mice reacted with E1 from RV virions under reducing conditions (Fig. 24, + β-Me). These results suggest that native immunogenic determinants of E1 glycosylation mutants expressed via vaccinia recombinants are predominantly conformation-dependent. This could be due to aberrant folding of mutant proteins and masking of linear epitopes on E1 when normal glycosylation is blocked.

To further assess the production of anti-E1 antibodies from mice immunized with vaccinia recombinants, an ELISA was used to quantitate the E1-specific antibodies of mouse sera (Table 2). The antibody titre from VR-E1 immunized mice was found to be three times higher than that in sera from those immunized with VR-E1G1, VR-E1G2, VR-E1G3 and VR-E1G23, and 15 times higher than that in serum from mice immunized with VR-E1G123. Table 2 shows the analysis of HAI and VN activities of antisera from mice immunized with vaccinia recombinant viruses. VN activity was observed in sera from mice immunized with VR-E1, VR-E1G1, VR
Fig. 24 Immunoblot analysis of sera from mice immunized with E1 vaccinia recombinants. Purified RV particles were subjected to SDS-PAGE under reducing (+ β-Me) and non-reducing (- β-Me) conditions, and transferred to nitrocellulose. E1 antigens were detected with antisera from mice immunized with E1 vaccinia recombinants. The immunizing vaccinia recombinants were indicated on the top of the gel. Wild-type vaccinia virus was used as a negative control (WT). E1-specific mAbs were used as a positive control (mAb). E1 monomer (•), E1-E2 heterodimer (○) and E1-E1 homodimer (*) were indicated. Molecular weight markers (in kDa) are shown on left for reference.
-E1G2 and VR-E1G3 (Table 2). Deletion of any single glycosylation site from E1 did not prevent neutralizing antibody production, as the ratio of VN titre to ELISA titre was similar in sera from mice immunized with VR-E1, VR-E1G1, VR-E1G2 and VR-E1G3 (Table 2). Thus, carbohydrate side chains on E1 are not directly involved in the elicitation of VN responses. HAI activities of these sera were examined with regard to capacity to block the binding of RV virions to erythrocytes. Sera from mice immunized with VR-E1, VR-E1G2 and VR-E1G3 showed HAI activity while that raised from VR-E1G1 did not (Table 2), suggesting that oligosaccharide attached at the G1 site is important in eliciting HAI antibody production. In contrast, no VN or HAI antibodies were detectable in the sera from mice immunized with either VR-E1G23 or VR-E1G123, indicating that VN and HAI epitopes were not functionally active in these mutant proteins. This may be due to an altered protein conformation when most of or all of the carbohydrate side chains are absent.

3.3.4. Antigenic properties of deglycosylated RV E1 from RV virions.

To further confirm the above conclusion, digestion of RV virions with endo F/PNGase F was performed under mild conditions which remove all N-linked oligosaccharides from proteins without disrupting protein conformation. The extent of deglycosylation and its possible effect on antigenicity were examined by immunoblotting (Fig.25). E1 from deglycosylated virions migrated as a 51 kDa protein [Fig.25, + β-Me, RV(dG)], similar to the non-glycosylated E1 expressed from vaccinia recombinant VR-E1G123 (Fig.25, + β-Me, VR-E1G123), indicating that all oligosaccharides attached to E1 had been removed (Fig.25, + β-Me). Under non-reducing
Table 2. Comparison of the HAI and VN antibodies from mice immunized with vaccinia recombinants containing different RV E1 glycosylation mutant cDNA inserts

<table>
<thead>
<tr>
<th>Virus used for immunization</th>
<th>ELISA titre&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HAI titre&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Comp.&lt;sup&gt;d&lt;/sup&gt;</th>
<th>No comp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV&lt;sup&gt;e&lt;/sup&gt;</td>
<td>512</td>
<td>128</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>VR-E1</td>
<td>128</td>
<td>64</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>VR-E1G1</td>
<td>128</td>
<td>64</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>VR-E1G2</td>
<td>128</td>
<td>64</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>VR-E1G3</td>
<td>128</td>
<td>64</td>
<td>8</td>
<td>4</td>
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<tr>
<td>VR-E1G23</td>
<td>128</td>
<td>64</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>VR-E1G123</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as the highest dilution of antibodies yielding OD₄₀₅ two times higher than background.
<sup>b</sup> Expressed as the highest dilution of antibodies that completely inhibits hemagglutination.
<sup>c</sup> Expressed as the end point of antibody dilution that completely inhibits plaque formation.
<sup>d</sup> Heat-inactivated rabbit complement (2.5%) was present in diluted antibodies.
<sup>e</sup> Wild-type vaccinia virus.
<sup>f</sup> For the technique employed in the assay, this is the lowest dilution of serum that can be achieved. Values under 16 were considered negative.
Fig. 25 Effect of deglycosylation on the antigenicity of RV virion. Untreated RV virion (RV), deglycosylated (dG), and cell lysate from VR-E1 and VR-E1G123 infected CV-1 cells were subjected to SDS-PAGE under reducing (+ β-Me) and non-reducing (- β-Me) conditions. Proteins were transferred onto nitrocellulose and RV E1-specific antigens were probed with E1-specific mAbs.
conditions, E1 from deglycosylated virions retained mAb binding activity, whereas non-glycosylated E1 from vaccinia recombinant VR-E1G123 had lost this activity (Fig.25, - β-Me). This suggests that removal of sugar moieties after protein folding has less effect than blocking glycosylation during protein synthesis prior to folding. The observed significant decrease in antigen mass for E1 from deglycosylated virions (Fig.25) could be due to aggregation of deglycosylated virions that pelleted during sample preparation. This observation was further confirmed by using deglycosylated E1 from RV virions as antigen in ELISA (Table 3). Using six RV E1-specific mAbs, I found that there was no difference in activity of binding to mAbs between the glycosylated and deglycosylated RV virions as determined in the ELISA (Table 3).

The role of carbohydrate in HA activity of E1 was examined with deglycosylated RV virions. Deglycosylation of RV virions resulted in a significant decrease in the HA titre (Table 3), suggesting that carbohydrate was functionally involved in hemagglutination. However, no difference was observed between the binding of glycosylated and deglycosylated virions to chick erythrocytes when HAI mAbs were used to inhibit the binding (Table 3), suggesting that the binding of HAI antibodies to E1 is carbohydrate-independent.

3.3.5. Effect of glycosylation on E1 cell surface expression.

To localize the subcellular distribution of the wild-type and mutant E1 proteins, CV-1 cells infected with recombinant vaccinia viruses were analyzed by indirect immunofluorescence (Fig.26). In cells infected with vaccinia E1 recombinants (wild-type and glycosylation mutants), E1 proteins were found in the ER and Golgi-like region with the exception of E1G123, which was found to show a more profound ER staining (Fig.26, a-d). It has been shown previously that
Table 3. A. HA assay of the deglycosylated RV virion

<table>
<thead>
<tr>
<th></th>
<th>Glycosylated</th>
<th>Deglycosylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA activity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>320</td>
<td>40</td>
</tr>
<tr>
<td>HA inhibition assay&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2560</td>
<td>2560&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3D5D</td>
<td>2560</td>
<td>2560&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>H4C52</td>
<td>5120</td>
<td>5120</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as the highest dilution of virus yielding hemagglutination.

<sup>b</sup>Expressed as the endpoint of antibody dilution that completely blocks the 4 HA units binding to chick erythrocytes (8-fold more deglycosylated viruses was used, compared to untreated virus).

B. Effect of deglycosylation of RV E1 on antibody recognition by E1-specific monoclonal antibodies.

<table>
<thead>
<tr>
<th>mAbs</th>
<th>ELISA titre&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Glycosylated</th>
<th>Deglycosylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>21B9H</td>
<td>256</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>H4C52</td>
<td>1024</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>3D5D</td>
<td>1024</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>14D1F</td>
<td>256</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>3D9F</td>
<td>256</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>16B2C</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as the highest dilution of antibodies yielding twice higher OD<sub>405</sub> than background.
E2 is essential for transport of E1 to the cell surface (Hobman et al., 1990). The effect of glycosylation on E1 cell surface expression was studied by infecting CV-1 cells with E1 vaccinia recombinant viruses (wild-type and glycosylation mutants), or with E1 vaccinia recombinant viruses plus RV E2 vaccinia recombinant viruses. No cell surface expression was detected in cells infected with vaccinia recombinants of either E1 wild-type or glycosylation mutants (data not shown). In cells co-infected with both E2 and E1 vaccinia recombinants, the internal distribution of E1 antigens remained unchanged (Fig.26, e-h), while cell surface expression of wild-type and single glycosylation mutant E1 was observed (Fig.26i, j). Cell surface staining was not detected in cells co-infected with either VR-E2 and VR-E1G23 or VR-E2 and VR-E1G123 (data not shown). The data presented here suggest that the cell surface expression of RV E1 requires at least two N-linked carbohydrate side chains on E1 proteins in addition to co-expression of RV E2.

3.3.6. Summary and Discussion

The role of N-linked glycosylation on the antigenicity and immunogenicity of E1 glycoprotein was studied using vaccinia recombinants expressing E1 glycosylation. The expressed E1 glycosylation mutant proteins were recognized by a panel of specific monoclonal antibodies in radioimmunoprecipitation, immunofluorescence and immunoblotting, indicating that carbohydrate side chains on E1 are not involved in the constitution of epitopes recognized by those monoclonal antibodies. This observation was further supported by the fact that there is no significant change in the antigenicity after oligosaccharides on E1 from virions were removed by glycosidase digestion. All glycosylation mutants were capable of eliciting anti-RV E1 antibodies at different
Fig. 26 Indirect immunofluorescence of E1 glycosylation mutants in infected CV-1 cells. For cell surface expression, cells were fixed with 3% formaldehyde and incubated with an E1-specific mAb mixture followed by the incubation with FITC-conjugated goat anti-mouse IgG. For detection of internal antigen, cells were permeabilized with 0.1% Nonidet-P40 prior to incubation with E1 mAbs. (a) VR-E1; (b) VR-E1G2; (c) VR-E1G23; (d) VR-E1G123; (e) VR-E1, VR-E2; (f) VR-E1G2, VR-E2; (g) VR-E1G23, VR-E2; (h) VR-E1G123, VR-E2; (i) VR-E1, VR-E2, surface; (j) VR-E1G2, VR-E2, surface. For glycosylation mutants G1 and G3, a similar staining pattern as G2 was observed (data not shown).
titres. The single glycosylation mutants (G1, G2 and G3), but not the double mutant or the triplemutant (G123), were found to be capable of inducing VN responses. However, among the single glycosylation mutants, only G2 and G3 were able to induce HAI antibodies in mice.

The influence of carbohydrate on the antigenicity of viral glycoproteins has been demonstrated in a number of viruses (see introduction section 1.1.3.4.). It has been suggested that oligosaccharide side chains mask adjacent polypeptide sequence and prevent the binding of respective antibodies. In our case, no difference in antigenicity was observed between the wild-type and mutant E1 proteins expressed from vaccinia recombinants, with respect to their mAb binding activities in immunoblotting under reducing conditions (Fig.23), suggesting that there are carbohydrate-independent epitopes in E1 for the six mAbs tested. Consistent with this interpretation, deglycosylation of RV virions with endo F/PNGase F resulted in no quantitative change in reactivity to E1-specific mAbs when analyzed by ELISA (Table 3). However, a significant decrease was observed in HA activity of the deglycosylated RV virions (Table 3). It is concluded that carbohydrate on E1 is not involved in the constitution of epitopes recognized by mAbs but is functional in hemagglutination. However, non-glycosylated E1 expressed from VR-E1G123 failed to react to any of the mAbs under non-reducing conditions, presumably due to aberrant folding of the mutant protein when carbohydrate side chains were absent.

Most viral glycoproteins contain multiple N-linked glycosylation sites. Site-specific effects on the processing and intracellular transport of glycoproteins have been reported for SV5 HN (Ng et al., 1990) and also observed for RV E2 (see 3.1. in this section). In this study, all the RV E1 mutants were found to reside in the ER and Golgi-like region except VR-E1G123 which was localized predominantly in the ER region. All three single glycosylation mutants (E1G1, E1G2,
E1G3) were found to be transported to the cell surface when RV E2 was present in the cells (Fig.26j), suggesting that carbohydrate at each of the three sites affects intracellular transport of E1 equally. It has been reported previously that co-expression of RV E2 is required for cell surface expression of RV E1, whereas E2 transport to the cell surface is an E1-independent event (Hobman et al., 1990). In this study, I have shown that besides co-expression of E2, cell surface expression of E1 also requires any two of the three N-linked oligosaccharides. Taken together, this suggests that the interaction of E1 and E2 is a post-translational event rather than a co-translational process and the formation of E1-E2 heterodimer is probably important to release E1 from retention in the Golgi apparatus.

I was interested in exploring the site-specific effect of glycosylation on the immunogenicity of RV E1. Immunization of mice with vaccinia recombinants expressing E1 glycosylation mutants showed that removal of any one of the carbohydrates from E1 does not prevent single mutants from inducing VN antibodies in mice, suggesting that the protective immune response is probably directly toward the polypeptide backbone. Similarly, neutralizing activity is also detected in rabbit serum raised against RV E1 peptide expressed in E.coli (Terry et al., 1989). These results are consistent with the finding that the VN epitopes of E1 map to a region of the C-terminal half of E1 that does not contain glycosylation sites (Terry et al., 1988; Wolinsky et al., 1991; Chaye et al., 1992a). The failure of VR-E1G23 and VR-E1G123 to elicit neutralizing antibodies is probably due to improper conformation of the mutant proteins. A considerably lower antibody response was found in sera from mice immunized with VR-E1G1, VR-E1G2, and VR-E1G3. Of three single mutants, VR-E1G1 did not induce HAI antibodies, indicating that oligosaccharide attached to the G1 site is either involved in hemagglutination directly or is critical for facilitating
hemagglutinin epitope exposure. The hemagglutinin epitopes of E1 have been defined as residing in the same region as neutralizing epitopes (Terry et al., 1988; Chaye et al., 1992a), a carbohydrate-free domain. However, I have shown that hemagglutination is carbohydrate-dependent (Table 3). Thus, the expression of HA epitopes for the induction of HAI antibody production is dependent on the conformation of native E1 protein. It has been shown that an E1 peptide expressed in E.coli is recognized by HAI mAbs but fails to produce HAI antibody in rabbits (Terry et al., 1988).

The results presented here indicate that although the addition of carbohydrate is not essential for antibody binding to E1, deletion of any one of the oligosaccharide side chains from E1 results in a less immunogenic state of E1, probably due to improper folding. Thus, in developing an effective RV subunit vaccine using E1, proper combinations of different epitopes in their immunoactive conformations must be considered.
3.4. Section IV. Expression and characterization of virus-like particles containing rubella virus structural proteins

3.4.1. Isolation of BHK cell lines expressing RV structural proteins

Three RV cDNA constructs (Fig.27) were used in the isolation of stably transformed BHK cell lines. These cDNAs encode the capsid protein (C), E2E1 polyprotein precursor (E2E1) or polyprotein precursor for all three structural proteins of RV (24S) (Clarke et al., 1987; Hobman et al., 1990). The cDNAs were subcloned into the Sma I site of transfer vector pNUT (Fig.4c) (Palmiter et al., 1987), under the control of the metallothionein I promoter (Fig.4c). The resultant recombinant plasmids were transfected into BHK cells using the calcium phosphate method (2.2.5.2) (Gorman et al., 1982). Twenty-four hours after transfection, methotrexate (2.5 mM) was added to the culture medium and cells were incubated with this selection medium for 10 days. Methotrexate-resistant colonies were picked and screened for the integration of RV cDNAs into their chromosomes using the polymerase chain reaction (Saiki et al., 1988) and for the expression of RV structural proteins using western blot analysis (Towbin et al., 1979). Isolated cell lines were stable under normal growth conditions as they retained the capacity to express RV structural proteins after four months of continuous culturing. Cell lines were named according to the RV cDNAs used for transfection, as BHK-C, BHK-E2E1 and BHK-24S, respectively.
Fig. 27 Diagrammatic representation of RV cDNAs used in the construction of recombinant plasmids. Translation initiation site (ATG) from RV capsid protein was used in all constructs. The putative signal peptides and membrane anchor domains of E2 and E1 are indicated as \[\text{ signal peptide }\] or \[\text{ membrane anchor }\], respectively.
3.4.2. Expression of RV structural proteins

Monolayers of stably transformed cells were incubated with medium containing 30 µM zinc sulfate for 12 hours to induce the expression of RV structural proteins from the promoter. The expression of RV structural proteins from stably transformed cell lines was analyzed by immunoblotting using human anti-RV serum (Towbin et al., 1979). In BHK-C cells, an intracellular protein species with molecular size of 34 kDa was observed (Fig. 28A, lanes C). This protein may represent the capsid protein of RV. In BHK-E2E1 cells, protein species corresponding to the ER- and Golgi- isoforms of RV E2 (Hobman et al., 1990) and E1 glycoproteins were found in the cell lysate but not in the medium (Fig. 28A, lanes E2E1), indicating that the E2E1 polyprotein precursor was synthesized and proteolytically processed to give rise to E2 and E1 proteins. In BHK-24S cells, protein species corresponding to the C, E2 and E1 proteins of RV were present in the cell lysate as well as in the medium (Fig. 28A, lanes 24S), suggesting that the integrated cDNA of 24S RNA was active in directing the synthesis of RV structural proteins and these structural proteins were released from the cells. The secretion of RV structural proteins from BHK-24S increased with time and was linear over a period of 24 hours under ZnSO₄ induction (Fig. 29).

3.4.3. Assembly and release of virus-like particles (VLPs) in stable BHK-24S cells

The secretion of RV structural proteins into the medium was found to be dependent on the coexpression of C, E2 and E1, suggesting that these proteins might be assembled into subviral particles prior to their release from the cells. To examine this possibility, medium from BHK-24S and RV-infected cells was subjected to ultracentrifugation (350,000xg for 20 minutes), in the
Fig. 28 A. Immunoblot analysis of proteins expressed in transformed BHK cells. Monolayers of BHK-C, BHK-E2E1 or BHK-24S cells were incubated with serum-free medium in the presence of 30 μM zinc sulfate for 12 hours. Culture media were collected and cell monolayers were lysed with RIPA buffer. Samples were directly subjected to SDS-PAGE and immunoblotting. B. Immunoblot analysis of proteins sedimented by ultracentrifugation. Samples from medium of induced BHK-24S (Lanes 1 and 2) or RV-infected BHK cells (Lane 3) were centrifuged at 90,000 rpm for 20 minutes in the absence (Lanes 2 and 3) or presence of 1% NP-40 (Lane 1). The pellets were resuspended in RIPA buffer and analyzed using SDS-PAGE and immunoblotting. The positions of RV structural proteins are indicated by arrowhead. The molecular weight markers are included for reference.
Fig. 29. Time course of VLPs secretion from BHK-24S cells. Expression of RV structural proteins was induced by the addition of ZnSO₄ (30 μM in the culture medium). Culture medium was collected and cells were lysed at the indicated times (hour post-induction). Samples from medium were subjected to 90,000 rpm centrifugation for 20 minutes and resuspended in RIPA buffer. The resuspended pellets and the cell lysates were analyzed using SDS-PAGE and immunoblotting. The positions of RV structural proteins are indicated.
presence or absence of 1% non-ionic detergent, Nonidet P-40. Resuspended pellets were subjected to SDS-PAGE, transferred to nitrocellulose membranes and probed with human anti-RV serum. In the absence of NP-40, C, E2 and E1 were detected in the pellets from BHK-24S and RV-infected cells (Fig.28B, lanes 2 and 3). In the presence of NP-40, E1 and E2 glycoproteins remained in the supernatant after ultracentrifugation (not shown), although trace amounts of C were found in the pellet (Fig.28B, lane 1). Thus the assembled viral proteins are secreted as particles that sediment in a gravitational field. To confirm that proteins E1, E2 and C assembled into VLPs, samples from pelleted VLPs were centrifuged for 16 hours at 90,000xg through a density gradient from 20 to 50% sucrose. VLPs were recovered in fractions with density of 1.17-1.19 g/ml (Fig.30); similar to that of native RV virion (1.175-1.20 g/ml) (reviewed by Horzinek, 1981).

3.4.4. Electron-microscopic analysis of the VLPs

The morphology of the VLPs was analyzed by employing conventional electron microscopic technique with routine Epon embedding of fixed BHK-24S cells. The VLPs found in BHK-24S cells were comparable in size to RV particles (60 nm) (Fig.31A) and indistinguishable in appearance, with an electron dense core surrounded by an envelope (Fig.31B, C, D). These particles were predominantly located within the vacuoles in the juxtanuclear region (Fig.31D) or cytoplasm (Fig.31C), which may represent the Golgi structure. Some particles were distributed in the cytoplasm (Fig.31B), not associated with any membrane structure. Such particles were not observed in BHK-E2E1 or BHK-C cells (data not shown). Taken together, it is evident that VLPs were indeed assembled intracellularly prior to their release from the cells.
Fig.30 Purification of VLPs and RV on sucrose density gradient centrifugation. Pelleted RV or VLPs from 35 ml culture medium were resuspended in 0.35 ml TNG buffer (50 mM Tris, pH7.5; 100 mM NaCl; 200 mM glycine) and applied onto the top of a 12 ml-sucrose gradient of 20-50% sucrose in TNG. Centrifugation was carried out using a Beckman SW41 rotor at 90,000xg for 16 hours at 15°C. Fractions (~ 0.5 ml/fraction) were collected by puncturing the bottom of the tube and the density of each fraction was determined. 100 μl sample from alternative fractions were diluted with equal amount of TNG buffer and subjected to centrifugation at 90,000 rpm for 20 minutes. The pellets were resuspended with RIPA buffer. RV proteins in the pellets, and in the samples that loaded onto the gradient (load), were analyzed by SDS-PAGE and immunoblotting (using human anti-RV serum). Fractions are numbered from the bottom (#1) to the top (#23) of the gradient. The positions of RV structural proteins are indicated.
Fig. 31 Electron microscopic analysis of the VLPs in BHK-24S cells. RV-infected BHK cells (A) or induced BHK-24S cells (B, C, D) were fixed with formaldehyde/glutaraldehyde, postfixed with osmium tetroxide, ethanol dehydrated and Epon embedded. Thin sections were analyzed by electron microscopy after staining.
3.4.5. Antigenicity of the VLPs.

HA activity of the VLPs was examined using a heparin/manganous chloride procedure (Liebhaber, 1970) and the HA titre was expressed as the endpoint of serial dilutions of sera at which erythrocyte aggregation was observed. The VLPs from BHK-24S cells displayed HA activity of 64, while RV particles retained HA activity when diluted to 1/32. This difference is due to the higher yield of VLPs from induced BHK-24S cells than that of RV from infected cells.

To compare the antigenicity of VLPs with that of RV, equal amounts of RV or VLPs (with respect to HA unit) were used in each assay. Table 4 shows the antibody binding activities of VLPs and RV in immunoblot and ELISA analysis using twelve mAbs against RV E1, E2 or C. Two of the E2 mAbs showed differences in western blotting between the VLPs and RV (Table 4) and VLPs displayed a higher ELISA titre with a mAb against C protein than did RV (Table 4). VLPs were also used in a solid phase immunoassay to measure the IgG response in humans. With 200 human serum samples, it was found that the correlation coefficient between the VLPs and whole RV antigens was 0.96 using a non-parametric regression analysis method (data not shown). This indicated that the antigenic determinants on the VLPs resemble those of authentic RV.

3.4.6. Immunogenicity of the VLPs.

To evaluate the immunogenic properties of the VLPs, we immunized mice (BALB/c, four in each group) with the VLPs, RV or soluble E1 protein expressed in transfected BHK cells (Gillam, unpublished results), respectively, with comparatively same amounts of antigens (equivalent to
Table 4. Immunoreactivity of the VLPs with RV-specific monoclonal antibodies.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Western blot(^a)</th>
<th>ELISA titre(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>VLP</td>
</tr>
<tr>
<td>H15C22(C)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H32C43(E1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21B9H(E1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3D5D(E1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14B1F(E1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3D9F(E1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16A10E(E1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E2-2(E2)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E2-4(E2)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E2-5(E2)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E2-6(E2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H46C64(E2)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) Monoclonal antibodies were used at a dilution of 1:100 for ascites fluid or 1:5 for tissue culture supernatant.

+, positive reactivity was detected.
-
, negative reactivity was detected.

\(^b\) Expressed as the highest dilution of antibodies yielding OD\(_{405}\) two times higher than background.
256 HA units). The presence of anti-RV antibodies in the sera of immunized mice was analyzed using radio-immunoprecipitation. As shown in Fig.32, mice immunized with the VLPs produced antibodies against all three structural proteins of RV (Fig.32, lane 3), as did mice immunized with RV (Fig.32, lane 4). Mice immunized with E1 protein also developed some anti-E1 antibody response (Fig.32, lane 2).

ELISA was used to quantify the antibody titres against each of the RV structural proteins, by using individual purified RV structural proteins expressed in Spodoptera frugiperda cells infected with baculovirus recombinants (Gillam, unpublished data) as antigens. In the sera from mice immunized with VLPs, a significantly higher anti-C antibody titre was found, whereas anti-E1 and E2 antibody titres were slightly lower (Table 5). The biological functions of these antibodies were analyzed. Sera from VLP-immunized mice displayed VN activities (Table 5) as determined by plaque reduction assays (Fukuda et al., 1987). HAI activities were also present in the sera from mice immunized with the VLPs, as well as in the sera from mice immunized with RV (Table 5). These results suggested that although VLPs were less active in inducing overall anti-E1 and E2 antibodies compared to RV, they induced the production of both VN and HAI antibodies.

We have also determined cell-mediated immune responses against RV in VLP-immunized mice in a lymphocyte proliferation assay (Chaye et al., 1992a; Ou et al., 1992). Lymphocyte proliferative responses of mice were determined in vitro by direct stimulation of lymphocytes with UV-inactivated RV or individual RV structural proteins (C, E2 and E1) purified from recombinant baculovirus infected insect cells. Lymphocytes from VLP-immunized mice responded strongly to UV-inactivated RV as well as to the individual RV structural proteins in
Fig. 32 Radioimmunoprecipitation of RV structural proteins expressed in COS cells. COS cells were transfected with pCMV5-24S (Hobman and Gillam, 1989), labeled with $^{35}$S-methionine and lysed. RV structural proteins were recovered from cell lysates with mouse anti-RV antibodies pre-bound to Sepharose 4B-protein A beads as previously described (Hobman and Gillam, 1989) and separated by SDS-PAGE. Sera were from mice immunized with E1 protein (Lane 2), VLPs (Lane 3), RV (Lane 4) or from pre-immune mice (Lane 1). The positions of RV structural proteins are indicated.
Table 5. Comparison of antibody titres of mouse sera from mice immunized with different RV antigens.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>ELISA titre to RV protein*</th>
<th>VN titre^b</th>
<th>HAI titre^c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>E2</td>
<td>E1</td>
</tr>
<tr>
<td>E1</td>
<td>&lt;10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>RV</td>
<td>40</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>VLP</td>
<td>320</td>
<td>40</td>
<td>80</td>
</tr>
</tbody>
</table>

*Individual RV structural proteins (C, E2 and E1) were purified from SF9 cells infected with baculovirus recombinants expressing each RV structural protein (unpublished data).
^bExpressed as the reciprocal of the highest antibody dilution that show 50% reduction in plaque formation.
^cExpressed as the reciprocal of the highest antibody dilution that inhibited hemagglutination.
Fig. 33 Lymphoproliferation responses of mice immunized with VLPs. Lymphocytes from VLP-immunized mice (2.5\(\times\)10^5/well) were incubated with different concentrations of proteins E1 (■), E2 (+) or C (*) at 37°C for 5 days before addition of \([^3]H\)-thymidine (1 μCi/well). All assays were performed in triplicate and results are expressed as mean values. Using UV-inactivated RV (10^8 PFU/ml), lymphoproliferation responses in the presence and absence of RV antigen were 23,000 and 2,000cpm respectively.
3.4.7. Summary and Discussion

Noninfectious VLPs containing three structural proteins were expressed in a BHK cell line (BHK-24S) by using an inducible promoter. These VLPs were found to resemble RV virions in terms of their size, morphology and some biological activities. In immunoblotting studies, VLPs were found to bind similarly to native RV virions with 10 of a panel of 12 RV-specific murine monoclonal antibodies. Immunization of mice with VLPs induced specific antibody responses against RV structural proteins as well as VN and HAI antibodies. After immunization of mice with VLPs, *in vitro* challenge of isolated lymphocytes with inactivated RV and individual RV structural proteins stimulated proliferation.

The assembly of RV virion involves at least two major steps: encapsidation and envelopment of nucleocapsid. In RV, encapsidation occur in the cytoplasm as newly synthesized capsid protein interacts with genomic RNA to form icosahedral nucleocapsids. The packaging of genomic RNA into the nucleocapsid is believed to be a specific event as the 40S genomic RNA but not the 24S subgenomic RNA is packaged into RV virion (Oker-Blom et al., 1984). Recently, a stretch of 31 nucleotides on the 5' end of the RV genome has been identified as responsible for the binding of the genomic RNA to the capsid protein *in vitro* (Liu et al., unpublished data). Employing reverse transcription combined with the polymerase chain reaction (Saiki et al., 1988), we failed to detect any RV-specific RNA in the pseudovirion secreted from stable BHK-24S cells (data not shown), suggesting that capsid proteins can interact with each other and form a nucleocapsid-like structure. The VLPs were found to have a higher ELISA titre with a C-specific mAb (Table 4) and to elicit stronger anti-C antibody response in mice than those from RV (Table
5), implying that the VLPs contain more C protein than RV either due to its relative amount or conformational exposure in the particles. Although the pseudovirions do not contain RV-specific RNA, we cannot rule out the possibility that they might package some cellular RNAs or even DNAs into the nucleocapsid.

Incorporation of nucleocapsid into the membrane envelope to form virus particles is a yet poorly understood event in virus assembly. We found that in BHK-C and BHK-E2E1 cells, no RV proteins were released into the medium (Fig.28A). In BHK-24S cells, all three structural proteins were present in the medium as the result of subviral particle formation and egress (Fig.28A). These data strongly suggest that the interaction between glycoproteins and the nucleocapsid is the driving force for the assembly and release of the VLPs. This interaction has been defined to occur between the cytoplasmic tail of glycoprotein E1 and the capsid protein. Deletion or substitution of the cytoplasmic domain of RV E1 abolished the delivery of the VLPs into the medium (Hobman et al., unpublished data).

Besides being a useful tool to study RV assembly, BHK-24S cells in which VLPs are steadily assembled and released can be used as a potential source for mass production of rubella antigens at low cost under inducing conditions. BHK-24S cells continuously produce VLPs for up to 5 days without cell lysis when 30 μM ZnSO₄ is present in the medium and up to one month in DME/F12 (GIBCO) medium. VLPs can be harvested daily from the medium, which is replaced with fresh medium after harvesting. Depending on the methods used to quantitate the yields of the VLPs and RV from culture medium, the yield of VLPs was found to be two-fold higher than RV in an HA assay, five times higher in ELISA assays using human sera, and more than ten times higher by protein quantitation using silver staining after gel electrophoresis (data
It has been proposed that immunogenicity can be achieved by presenting antigens on a polyvalent particle structure. This concept led to the development of chimeric virus (Clarke et al., 1987; Michel et al., 1988; Li et al., 1993), virus-like particles (Griffiths et al., 1993) or immunostimulating complexes (Takahashi et al., 1990), in which multiple copies of antigen are integrated in a particulate form. These particles have been found to induce both humoral and cell-mediated immune responses, including VN antibodies (Michel et al., 1988; Griffiths et al., 1993; Li et al., 1993), T helper cells or cytotoxic T lymphocytes (Takahashi et al., 1990; Griffiths et al., 1993; Li et al., 1993) in animals. In our case, the VLPs were found to be significantly more active than the soluble E1 protein in inducing antibody responses in mice, especially for the production of VN and HAI activity (Table 5). The VLPs also evoked cell-mediated immune response to RV and RV structural proteins. This is believed to be important in providing protective immunity against RV infection. Preliminary results have shown that CD4+ T cells may be the major effector in cell-mediated immune responses elicited by the VLPs in mice, whereas CD8+ T cells may be also involved (data not shown). A study of the phenotype of the effector cells in proliferation assays is in progress. The VLPs are composed of all three structural proteins of RV, which make them similar to RV regarding antigen-presentation. These studies suggest that the noninfectious but highly immunogenic VLPs may serve as a candidate for safe vaccine development.
4. SUMMARY and PERSPECTIVES

In the Togavirus family, RV bears a striking similarity to the prototype alphaviruses (SFV and SIV) in terms of genomic organization and strategy for gene expression. However, RV research has fallen far behind that of alphaviruses due to the fact that RV has a much slower replication kinetics and limited cytopathological effects, and there has been limited success in producing large numbers of monoclonal antibodies directed to RV structural proteins (C, E2). In recent years, with the aid of recombinant DNA technology and mammalian expression systems, much progress has been made in studies on the expression, processing and biological functions of RV structural proteins. In this study, I have attempted to employ various mammalian expression systems (COS cell transient expression, vaccinia recombinant virus, stably transformed cells and RV-infected cells), combined with recombinant DNA techniques (including site-directed mutagenesis), to explore the structure/function relationship of RV structural proteins.

The role of N-linked glycosylation of RV E1 and E2 has been studied with respect to their biological function in RV maturation. RV E1 is the dominant surface molecule of the RV virion and the major target for human immune surveillance. The influence of N-linked glycosylation on antigenicity and immunogenicity of RV E1 has been investigated by expressing E1 glycosylation mutant proteins via vaccinia recombinants and by analyzing the immunoreactivity of deglycosylated E1 protein from RV virions treated with glycosidase to remove carbohydrates on E1. It appears that all three N-linked glycosylation sites on E1 are required to maintain an optimal protein configuration for the exposure of epitopes determining biological functions (i.e. neutralization and hemagglutination). Deletion of any of the N-linked glycosylation sites on E1 results in reduction of antibody production and this effect seems to be additive. Carbohydrates
on E1, important for the hemagglutination activity of the virion, however, are not involved in the constitution of the epitopes recognized by monoclonal antibodies used in this study.

Unlike E1, the biological function of E2 is not clear except that it is believed that in RV-infected cells, E2 serves as a carrier to deliver E1 from a post-ER, pre-Golgi compartment to the Golgi complex. The role of N-linked glycosylation on intracellular transport and processing of RV E2 has been analyzed by transient expression of E2 glycosylation mutant proteins in COS cells. N-linked glycosylation at all three sites is essential for transport competence of E2. Deletion of any glycosylation site on E2 leads to a significant reduction in rate of glycan processing, lower stability and retention of mutant proteins in the ER.

A common effect of deleting glycosylation sites between E1 and E2 is the formation of aberrant disulfide bonds. Amino acid sequences predicted from cDNA sequences indicate that both E1 and E2 are rich in cysteine residues. The implication of this is that proper folding and correct disulfide bonding may be a slow process, sensitive to modulation by structural alteration or environmental influence. Intra- and intermolecular disulfide bonds have been observed in wild-type, as well as mutant E1 and E2 when they are expressed separately (Fig.9, Fig.23). It appears that deletion of glycosylation sites adjacent to a cysteine residue (E1G1, E2G2 and E2G3) has a more profound deleterious effect. Therefore, it is conceivable that the addition of carbohydrate contributes to the proper folding and subsequent correct disulfide bonding, which may be critical for the biological functions of E1 and E2.

Although the role of N-linked glycosylation on the functions of E1 and E2 has been studied in some detail, the effect of glycosylation during RV replication and infection is still largely unknown. Deglycosylated RV virions lost hemagglutination activity (Table 3) while their
infectivity has not been determined. Recently, Dr. T.K. Frey and co-workers have succeeded in the construction of RV genome-length cDNA clones. Transfection of cells with RNA transcribed from one of these clones results in the production of virus that preserved the genetic and phenotypic characteristics of the parental virus from which the cDNA clone was derived. Such an infectious cDNA clone will facilitate the evaluation of site-specific effect of glycosylation in viral assembly and infectivity (Wang et al., 1994).

Due to the fact that RV infectious cDNA was not available during this study, an alternative approach was taken to elucidate the structure/function relationship of RV structural proteins during virus assembly. Tunicamycin, BFA and monensin were used to inhibit or to induce an altered processing and transport of RV glycoprotein in cDNA-transfected cells and RV-infected cells. Protein processing and transport as well as virus assembly and release were analyzed. BFA and monensin dramatically reduce the assembly of intracellular infectious virions while tunicamycin completely inhibit virus assembly. The effect of tunicamycin, BFA and monensin on virus infectivity parallels that of the disruption of distribution of E1 and E2 in an intact Golgi complex, which points out the possibility that probably a stable association of E1 and E2 with the Golgi structure may be essential for efficient assembly of RV. This is in a good agreement with the fact that RV E1 and E2 are found to be targeted to the Golgi complex (Hobman et al., 1993) with a limited amount expressed at the cell surface (Hobman et al., 1990). In RV-infected cells, the released virus has a slightly higher virus titre than that of intracellular virus (even during the early stage of virus life cycle), and is found to contain O-linked glycans as well as complex-type of N-linked sugars on E2. The intracellular viruses has not been purified to homogeneity or distinguished from unassembled RV structural proteins due to technical
difficulties, therefore, the detailed oligosaccharide structures on E1 and E2 of intracellular virions are not known. It is possible, however, that the difference in the status of glycan maturation between extra- and intracellular viruses may contribute to their infectivity. In addition, further studies are necessary to define the mechanism of retention of RV glycoprotein in the Golgi apparatus, and the importance of Golgi-specific modifications (i.e. O-linked glycosylation) on virus assembly.

Three stably transformed cell lines expressing RV structural proteins have been constructed. RV structural proteins are found to assemble into virus-like particles in the Golgi complex prior to their release from the cells. The assembly of VLPs is found to be independent of genomic RNA but is strictly dependent upon co-expression of all three structural proteins. The VLPs resemble RV virions in terms of size, buoyant density, morphological appearance and protein composition. These observations point out the future direction in studying the structure/function relationship and protein-protein interaction during RV assembly. Stable cell lines expressing RV structural proteins in different combinations under an inducible promoter, or with molecular modifications, will be a useful system.

The non-infectious VLPs have been found to bind similarly to native RV virions with RV-specific monoclonal antibodies as well as human sera. Humoral (including viral neutralizing and hemagglutination inhibition) and cell-mediated immune responses have been detected in mice immunized with the VLPs. Therefore, the VLPs may serve as a convenient source of RV antigen for serodiagnostic assays and a potential candidate for vaccine development. The VLPs contains all three RV structural proteins and closely resemble RV regarding antigen presentation, having the advantage over synthetic peptide or subunit vaccines containing single species of RV
structural proteins. However, it should be noted that since it has been suggested that molecular mimicry of E2 (Yoon et al., 1992) and C (Karounos et al., 1993) may lead to autoimmune tissue damage, such epitopes should be deleted from the VLPs for safer vaccine development.
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